

FORM PCT 1536
(Rev. 1-98)
U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

**TRANSMITTAL LETTER TO THE UNITED STATES
DESIGNATED/ELECTED OFFICE (DO/EO/US)
CONCERNING A FILING UNDER 35 U.S.C. 371**

ATTORNEY'S DOCKET NUMBER JAB-1526

JC20 Rec'd PCT/PTO 20 FEB 2002

U.S. APPLICATION NO. (If known, see 37 CFR 1.5)

10/069495

INTERNATIONAL APPLICATION NO.

PCT/EP00/08182

INTERNATIONAL FILING DATE

August 21, 2000

PRIORITY DATE CLAIMED

August 24, 1999

TITLE OF INVENTION

GAS1 POLYPEPTIDES

APPLICANT(S) FOR DO/EO/US :

Luyten, Walter H.M.L.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
 - ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
 - ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
 - a. ☒ is transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☐ has been transmitted by the International Bureau.
 - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
 - ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
 - ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
 - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☐ have been transmitted by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☐ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).
9. ☒ An unexecuted oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ A translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A FIRST preliminary amendment.
 - ☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☒ A substitute specification. Modified with U.S. headings
15. ☐ A change of power of attorney and/or address letter.
16. ☒ Other items or information: Express mail certificate, copy of the International Preliminary Examination Report, copy of the International Search, Sequence Disk, Sequence Listing, Version to show changes made and post card

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PCT/PTO 24 FEB 2002

17. ☒ The following fees are submitted:

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):

Neither international preliminary examination fee (37 CFR 1.482)
nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO
and International Search Report not prepared by the EPO or JPO.....\$1040.00

International preliminary examination fee (37 CFR 1.482) not paid to
USPTO but International Search Report prepared by the EPO or JPO.....\$890.00

International preliminary examination fee (37 CFR 1.482) not paid to
USPTO but international search fee (37 CFR 1.455(a)(2)) paid to USPTO..... \$740.00

International preliminary examination fee (37 CFR 1.482) paid to
USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4)..... \$750.00

International preliminary examination fee (37 CFR 1.482) paid to
USPTO and all claims satisfied provisions of PCT Article 33(1)-(4)..... \$100.00

ENTER APPROPRIATE BASIC FEE AMOUNT =

CALCULATIONS PTO USE ONLY

\$ 1040.00

Surcharge of \$130.00 for furnishing the oath or declaration later than ☐ 20 ☐ 30
months from the earliest claimed priority date (37 CFR 1.492(e)).

\$

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE
Total claims	49 - 20 =	29	x \$18.00
Independent claims	19 - 3 =	16	x \$84.00
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ \$280.00

\$ 522.00

\$ 1,344.00

\$

TOTAL OF ABOVE CALCULATIONS =

\$ 1,866.00

Reduction of 1/2 for filing by small entity, if applicable. A Small Entity Statement
must also be filed (Note 37 CFR 1.9, 1.27, 1.28).

\$

SUBTOTAL =

\$2,906.00

Processing fee of \$130.00 for furnishing the English translation later than ☐ 20 ☐ 30 \$
months from the earliest claimed priority date (37 CFR 1.492(f)).

\$

TOTAL NATIONAL FEE =

\$

Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be
accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property +

\$ 40.00

TOTAL FEES ENCLOSED =

\$2,946.00

Amount to be
refunded:

\$

charged:

\$2,946.00

a. ☐ A check in the amount of \$ _____ to cover the above fees is enclosed.

b. ☒ Please charge my Deposit Account No. 10-0750/JAB-1526/MHM in the amount of \$2,946.00 to cover the above fees.
A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any
overpayment to Deposit Account No. 10-0750/JAB-1526/MHM. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a)
or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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Signature

Myra H. McCormack
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JC19 Rec'd PGT/PTO 20 FEB 2002

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants : Luyten, et al.

Serial No. :

Art Unit: UNKNOWN

Filed : herewith

Examiner: UNKNOWN

For : GAS1 Polypeptides

I hereby certify that this correspondence is being deposited with the
United States Postal Service as first class mail in an envelope addressed
to: Commissioner for Patents, Washington, D.C. 20231 on

February 20, 2002

(Date)

Myra H. McCormack

Name of applicant, assignee, or Registered Representative



(Signature)

February 20, 2002

(Date of Signature)

Hon. Commissioner for Patents
Washington, D.C. 20231

PRELIMINARY AMENDMENT

Dear Sir:

Prior to the examination of the above-referenced
application, kindly amend the application as follows:

IN THE SPECIFICATION

Kindly insert a copy of the Sequence Listing as
provided herewith.

Kindly add the following paragraph after the title
of the application:

--This application is a national stage application
of PCT/EP00/08182 filed August 21, 2000 entitled "Gas 1
Polypeptides" which claims priority from EP 99306702.4

entitled "Gas1 Polypeptides" filed August 24, 1999, the contents of these applications is incorporated by referenced into this application.--

IN THE CLAIMS

Kind cancel claims 19-21,46 and 47.

Kindly amend the following claims to read:

1. (Amended) A method of inhibiting the lethal effect of expressing an otherwise lethal protein in a cell, said method comprising the steps of:

- (a) providing a cell, tissue or organism having
 - (i) a nucleotide sequence encoding a Gas1 protein, or a functional equivalent, derivative or bioprecursor thereof, which is capable of inducing apoptosis in said cell and
 - (ii) a further nucleotide sequence encoding a protein which is otherwise lethal to said cell in itself or in response to a lethal stimulus in the presence of Gas1;
- (b) inhibiting function and/or expression of said Gas1 protein or functional equivalent, derivative or bioprecursor thereof; and
- (c) expressing said sequence encoding said otherwise lethal protein

wherein said protein normally induces the expression or activates either Gas1 protein or a protein in the signal transduction pathway of which Gas1 is a component.

2. (Amended) A method of identifying compounds which inhibit or enhance expression or activity of proteins which are lethal to a cell, tissue or organism said method comprising the steps of:

- (a) providing a cell, tissue or organism comprising a nucleotide sequence encoding a Gas1 protein or a functional equivalent,

derivative or bioprecursor thereof, which is capable of inducing apoptosis in said cell, and ii) a further sequence encoding a protein which is otherwise lethal to said cell in itself or in response to a lethal stimulus in the presence of Gas1;

- (b) inhibiting function and/or expression of said Gas1 protein or functional equivalent, derivative or bioprecursor thereof or a protein in the apoptotic pathway of which Gas1 is a component;
- (c) expressing said sequence encoding said otherwise lethal protein;
- (d) contacting said cell with a compound to be tested; and
- (d) monitoring the effect of said compound on said otherwise lethal protein compared to an identical cell which has not been contacted with said compound

wherein said proteins normally induce the expression or activate either the Gas1 protein or a protein in the signal transduction pathway of which Gas1 is a component.

3. (Amended) A method according to claim 2 wherein said expression or activity of Gas1 protein is inhibited by providing a nucleic acid molecule in said cell which is capable of hybridising to mRNA corresponding to Gas1 DNA to prevent expression thereof.

4. (Amended) A method according to claim 2 wherein said expression or activity of said Gas1 protein is inhibited by inhibiting the expression or activity of a protein in the pathway of which Gas1 is a component.

5. (Amended) A method according to claim 2 wherein said cell is induced to express said Gas1 protein by contacting said cell with a stimulus that increases intracellular calcium levels in said cell.

7. (Amended) A method according to claim 2 wherein said further sequence encoding said otherwise lethal protein is expressed by providing it on a suitable expression vector.

8. (Amended) A method according to claim 2 wherein said lethal protein is a highly expressed recombinant protein.

9. (Amended) A method according to claim 2 wherein said otherwise lethal protein comprises any of a glutamate, NMDA, AMPA or kainate receptor.

11. (Amended) A method according to claim 3 wherein said nucleic acid molecule is provided as an oligonucleotide or as a vector including a nucleotide sequence of said nucleic acid molecule.

14. (Amended) A method according to claim 2 wherein said Gas1 protein is of mammalian origin.

16. (Amended) A method according to claim 14 wherein said Gas1 protein comprises the amino acid sequence depicted in either of Sequence ID No. 2 or 4 or a functional equivalent, derivative or bioprecursor thereof.

17. (Amended) A compound identifiable as an inhibitor or an enhancer of expression or activity of

an otherwise lethal protein according to the method of claim 2.

22. (Amended) The method of claim 54 wherein said cell is associated with a disease condition selected from the group consisting of a neurological disorder, a cardiovascular disorder, an autoimmune disorder, a neuroendocrine disorder or cancer.

26. (Amended) A nucleic acid molecule according to claim 25 which is a DNA sequence.

28. (Amended) A nucleic acid molecule according to claim 26 comprising the sequence of nucleotides according to Sequence ID No. 1.

29. (Amended) An antisense molecule capable of hybridising to the nucleic acid molecule of claim 25 under conditions of high stringency.

31. (Amended) A protein encoded by the nucleic acid molecule of Sequence ID NO. 1 or a nucleic acid molecule capable of hybridizing to Sequence ID NO.1 or its complement under conditions of high stringency.

32. (Amended) A protein comprising the amino acid sequence illustrated in Sequence ID No. 2.

34. (Amended) An expression vector comprising a nucleic acid molecule according to claim 25 or a nucleic acid molecule capable of hybridizing to Sequence ID No.1 under conditions of high stringency.

36. (Amended) An expression vector according to claim 34 comprising a tissue or cell specific promoter.

37. (Amended) An expression vector according to claim 34 further comprising a sequence encoding a proapoptotic protein.

38. (Amended) An expression vector according to claim 34 which is inducible for expression of a protein comprising the amino acid sequence of Sequence ID No.2 or a polypeptide capable of inducing apoptosis in a cell.

40. (Amended) A host cell, tissue or organism, transformed, transfected or infected with a vector according to claim 34.

42. (Amended) A method according to claim 41 wherein said cell in step (a) comprises a host cell, tissue or organism transformed, transfected or infected with a vector comprising a nucleic acid molecule encoding a protein capable of inducing apoptosis in a cell comprising an amino acid sequence according to Sequence ID No. 4 or a nucleic acid molecule capable of hybridizing to Sequence ID No.1 under conditions of high stringency.

43. (Amended) A compound identifiable as an inhibitor or an accelerator of cell death according to the method of claim 42.

45. (Amended) A pharmaceutical composition comprising any of a nucleic acid molecule encoding a protein according to Sequence ID No. 4 or a nucleic acid molecule capable of stringent hybridization thereto; an antisense molecule capable of hybridizing to a nucleic acid molecule encoding a protein according to Sequence ID No. 4 under conditions of high stringency; or a protein encoded by a nucleic

acid molecule capable of hybridizing to Sequence ID No. 1 under stringent hybridization conditions together with a pharmaceutically acceptable carrier, diluent or excipient therefor.

48. (Amended) The method of claim 22, wherein said neurological disorder is any of, Parkinson's disease, Alzheimer's disease, Huntington's disease, amyotrophic lateral sclerosis, a neurological condition caused by thrombosis or cerebral trauma.

49. (Amended) The method of claim 22, where said cardiovascular disorder is a heart attack.

50. (Amended) The method of claim 22, wherein said autoimmune disorder is multiple sclerosis.

51. (Amended) The method of claim 22, wherein said neuroendocrine disorder is necrosis of the pituitary gland.

52. (Amended) An antibody capable of binding to a protein comprising an amino acid sequence according to Sequence ID NO.2 or Sequence ID No. 4.

Kindly add the following claim:

54. A method for decreasing the expression of a protein that is lethal to a cell, wherein the protein normally induces the expression or activation of either the Gas1 protein or a protein in the signal transduction pathway of which Gas1 is a component comprising the step of: contacting the cell with a therapeutically effective dose of a compound identified by (a) providing a cell, tissue

or organism comprising a nucleotide sequence encoding a Gas1 protein or a functional equivalent, derivative or bioprecursor thereof, which is capable of inducing apoptosis in said cell, and ii) a further sequence encoding a protein which is otherwise lethal to said cell in itself or in response to a lethal stimulus in the presence of Gas1;


- (b) inhibiting function and/or expression of said Gas1 protein or functional equivalent, derivative or bioprecursor thereof or a protein in the apoptotic pathway of which Gas1 is a component;
- (c) expressing said sequence encoding said otherwise lethal protein;
- (d) contacting said cell with a compound to be tested; and
- (e) monitoring the effect of said compound on said otherwise lethal protein compared to an identical cell which has not been contacted with said compound.

REMARKS

Claims 1-5, 7-9, 11, 14-17, 22, 28, 29, 31, 32, 34, 36-38, 40, 42, 43, 45, 48-52 have been amended for U.S. patent practice. Claims 19-21, 46 and 47 have been canceled and claim 54 has been added as a rewrite of claim 20. Applicants submit a sequence listing along with a Computer Readable Form of the Sequence Listing. The undersigned hereby states that the Paper Copy and the Computer Readable Form are identical. The copy of the patent application included with this filing has been amended to introduce SEQ ID NOS for the oligonucleotides. No new matter has been added by

these amendments. Favorable consideration of this application is respectfully requested.

Respectfully submitted,


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Dated: February 20, 2002

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GAS1 POLYPEPTIDES

The present invention is concerned with methods of identifying compounds capable of preventing or accelerating Gas1 mediated cell death.

- 5 The Gas1 gene encodes a membrane protein which has been associated with the G0 phase of proliferative arrest and cell cycle exit in rat fibroblasts deprived of serum. Based upon its antiproliferative effects and its functional dependency to p53, Gas1 has also
- 10 been associated with antitumour like activity. (Evdokiou A. and Cowled P.A., 1998, Exp.Cell Res. (240), 359-367; Schneider et al., 1988 Genes specifically expressed at growth arrest in mammalian cells, Cell 54:787-793; Del Sal et al, 1992, the
- 15 growth arrest specific gene, Gas1, is involved in growth suppression, Cell 70:593-607; Del Sal et al, 1994. Structure, function and chromosome mapping of the growth suppressing human homologue of the murine Gas1 gene, Proc. Natl. Acad. Sci. USA 91; 1848-1852).
- 20 The structural conformation of the Gas1 protein deduced from the amino acid sequence thereof, indicates the presence of two transmembrane segments. Except for a hypothetical RGD domain (Arginine-Glycine-Aspartic acid) which is known to interact with
- 25 integrines, Gas1 does not show any described domain that relates to its cell cycle arrest function. Recently, mRNA Gas1 induction during involution of the prostate, mammary gland and the ovarian luteal body, as a result of castration, lactation and birth
- 30 delivery arrest, respectively, has been observed (Jaggi et al., 1996, Regulation of a Physiological Apoptosis: Mouse mammary Involution. J. Davy Sci. 79: 1074-1084).
- 35 From expression studies, the present inventors have surprisingly found that Gas1 overexpression induces cell death in various cell types, such as, neurons and

AMENDED SHEET

EMPFANGSZETT 0. NOV. 1:10

AUSGABEZETT 0. NOV. 7:22

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neuroblastoma cell lines and that Gas1 is responsible for the induction of apoptotic activity in a cell.

Based upon the surprising relationship of Gas1 expression and apoptosis the present inventors have developed methods to study the effects of expressing proteins in a cell which are normally lethal to the cell by inhibiting expression or activity of either the Gas1 protein or a protein in the signal transduction pathway of which Gas1 is a component. These methods can be further applied to identifying compounds which inhibit or enhance the expression of those otherwise lethal proteins. An assay has also been developed to identify compounds which are capable of preventing or accelerating Gas1 mediated cell death.

Therefore in accordance with a first aspect of the invention there is provided, a method of inhibiting the lethal effect of expressing an otherwise lethal protein in a cell, said method comprising (a) providing a cell, tissue or organism having (i) a nucleotide sequence encoding a Gas1 protein, or a functional equivalent, derivative or bioprecursor thereof, which is capable of inducing apoptosis in said cell and (ii) a further nucleotide sequence encoding a protein which is otherwise lethal to said cell in itself or in response to a lethal stimulus in the presence of Gas1; (b) inhibiting function and/or expression of said Gas1 protein or functional equivalent, derivative or bioprecursor thereof or a protein in the apoptotic pathway of which Gas1 is a component; and (c) expressing said sequence encoding said otherwise lethal protein.

35

Thus, advantageously, it is now possible, by inhibiting the function or expression of the

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biological mediator of cell death to study proteins which are normally lethal when expressed in a cell. These methods can also be used to identify compounds which can function as enhancers/inhibitors of expression or activity of the otherwise lethal proteins and which has not been possible hitherto.

Therefore, according to a second aspect of the present invention there is provided a method of identifying compounds which inhibit or enhance expression or activity of proteins which are otherwise lethal to a cell, tissue or organism said method comprising (a) providing a cell, tissue or organism comprising a nucleotide sequence encoding a Gas1 protein or a functional equivalent, derivative or bioprecursor thereof, which is capable of inducing apoptosis in said cell, and ii) a further sequence encoding a protein which is otherwise lethal to said cell in itself or in response to a lethal stimulus in the presence of Gas1; (b) inhibiting function and/or expression of said Gas1 protein or a functional equivalent, derivative or bioprecursor thereof or a protein in the apoptotic pathway of which Gas1 is a component; (c) expressing said sequence encoding said otherwise lethal protein; (d) contacting said cell with a compound to be tested; and (e) monitoring the effect of said compound on said otherwise lethal protein compared to an identical cell which has not been contacted with said compound.

Preferably, the inhibition of activity or expression of the Gas1 protein occurs by providing a nucleic acid molecule, such as an antisense molecule, which is capable of hybridising to mRNA in the cell corresponding to or complementary to Gas1 DNA under stringent conditions, to prevent expression thereof. The nucleic acid molecule in addition to possessing

antisense activity, may in some embodiments possess ribozyme or DNzyme activity.

5 The methods of the present invention, therefore, involve inhibiting the function or expression of a Gas1 protein *in vivo* using, for example, antisense technology. Antisense technology can be used to control gene expression through triple-helix formation or antisense DNA or RNA, both of which methods are
10 based on binding of a polynucleotide to DNA or RNA. For example, the part of the DNA sequence coding for the mature protein of the present invention is used to design an antisense RNA oligonucleotide of from 10 to 50 base pairs in length. A DNA oligonucleotide is
15 designed to be complementary to a region of the gene involved in transcription (triple-helix - see Lee et al. Nucl. Acids. Res., 6:3073 (1979); Cooney et al., Science, 241:456 (1988); and Dervan et al., Science, 251: 1360 (1991), thereby preventing transcription and the production of Gas1. The antisense RNA
20 oligonucleotide hybridises to the mRNA *in vivo* and blocks translation of an mRNA molecule into the mature protein. Thus, an animal having expression of the Gas1 protein inhibited may be utilised as a model for expression of otherwise lethal proteins in accordance
25 with the methods of the invention and for identifying potential therapeutic agents capable of inhibiting or enhancing expression or activity of the otherwise lethal proteins.

30 Preferably, the antisense molecule comprises a specific Gas1 antisense oligonucleotide (SEQ ID 5) which the present inventors have confirmed as being able to block the transduction process of the Gas1
35 protein and, as described in more detail below, involves a total blocking of the NMDA induced neuron death phenomenon in primary neuron cultures (survival

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practically of 100%, 25mM Ag1 column in Fig. 2a) or by staurosporin (200nM) (Fig. 2b). Alternatively, a sequence complementary to the nucleotide sequence encoding Gas1 as identified in SEQ ID No. 3 may be
5 used to prevent expression of the Gas1 protein.

In an even further embodiment it is also possible to inhibit Gas1 expression or activity by, for example, inhibiting expression or activity of a protein which
10 is involved in the pathway of which Gas1 is a component. Thus, using the methods described it is possible to prevent the apoptotic properties of Gas1 proteins being realised either by inhibiting, for example, the signal transduction pathway upstream or
15 downstream of Gas1 DNA to prevent the apoptotic response. It has also been identified that induction of Gas1 leads to a simultaneous increase in calcium concentration within the cell. In the method according to the invention a suitable stimulus can be
20 applied which is lethal to a cell, to induce transcription of the Gas1 protein.

In one embodiment of the methods described herein, the otherwise lethal protein may be provided on a suitable
25 expression vector. Suitable vectors are well known to those of skill in the art. Similarly, the sequence of the further nucleotide sequence encoding said otherwise lethal protein may be provided in an expression vector under the control of suitable
30 regulatory control elements. Such lethal proteins include, for example, glutamate receptors such as any of the type 1 to 8 metabotropic receptors, or NMDA, AMPA or kainate receptors. Alternatively, the lethal protein may comprise a highly expressed recombinant
35 protein which can frequently be toxic to a cell within which it is expressed. Thus, the present invention also, advantageously, provides a system to enable

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harvesting of highly expressed recombinant proteins.

The nucleic acid molecule utilised in accordance with the methods of the invention to inhibit expression of the Gas1 protein may be provided as an oligonucleotide, which is transformed or transfected into the cell, using techniques well known to those of skill in the art. Alternatively, the sequence may be encoded by a suitable sequence provided in a vector, which is transformed or transfected into the cell, tissue or organism.

Any compounds identified are also encompassed within the scope of the present invention. Thus, a further aspect of the invention provides a compound identifiable as an inhibitor or an enhancer of expression or activity of an otherwise lethal protein according to the invention. Such compounds may, for example, be included, in pharmaceutical compositions together with a pharmaceutically acceptable carrier, diluent or excipient therefor.

Compounds identified as enhancers of expression or activity may be used in the manufacture of a medicament for treating a disease condition mediated at least in part by underexpression or reduced activity of said otherwise lethal protein or a protein in the pathway of which said otherwise lethal protein is a component. Likewise compounds identified as inhibitors of expression or activity may be used in the manufacture of a medicament for treating a disease condition mediated at least in part by overexpression or reduced activity of said otherwise lethal protein or a protein in the pathway of which said otherwise lethal protein is a component. Preferably, the disease condition to be treated comprises any of a neurological disorder, a cardiovascular disorder, an

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autoimmune disorder, a neuroendocrine disorder or cancer.

These compounds can be used to prevent cell death in neurological disorders, such as for example, Parkinson's disease, Alzheimer's disease, Huntington's disease, amyotrophic lateral sclerosis, cerebellar ataxias, spinal muscular atrophies, etc.; stroke; head trauma; epilepsies; cardiovascular disorders, for example, post infarction, etc.; neuroendocrine disorders, for example, pituitary necrosis, etc.; autoimmune diseases, for example, multiple sclerosis, etc.; and any pathological process which is at least partially mediated by Gas1.

Furthermore, the present invention relates to a method of producing an antagonist or agonist of Gas1 according to the invention comprising the steps of any one of the above described screening methods; and additionally (i) synthesizing the compound obtained or identified in said method or a physiologically acceptable analog or derivative thereof in an amount sufficient to provide said antagonist or agonist in a therapeutically effective amount to a patient; and/or (ii) combining the compound obtained or identified in said method or an analog or derivative thereof with a pharmaceutically acceptable carrier.

The compounds isolated by the above methods also serve as lead compounds for the development of analog compounds. The analogs should have a stabilized electronic configuration and molecular conformation that allows key functional groups to be presented to the Gas1 protein in substantially the same way as the lead compound. In particular, the analog compounds have spatial electronic properties which are comparable to the binding region, but can be smaller

5 molecules than the lead compound, frequently having a
molecular weight below about 2 kD and preferably below
about 1 kD. Identification of analog compounds can be
performed through use of techniques such as self-
consistent field (SCF) analysis, configuration
interaction (CI) analysis, and normal mode dynamics
analysis. Computer programs for implementing these
techniques are available; e.g., Rein, Computer-
Assisted Modeling of Receptor-Ligand Interactions
10 (Alan Liss, New York, 1989). Methods for the
preparation of chemical derivatives and analogues are
well known to those skilled in the art and are
described in, for example, Beilstein, Handbook of
Organic Chemistry, Springer edition New York Inc., 175
Fifth Avenue, New York, N.Y. 10010 U.S.A. and Organic
15 Synthesis, Wiley, New York, USA. Furthermore, said
derivatives and analogues can be tested for their
effects according to methods known in the art; see
also supra. Furthermore, peptidomimetics and/or
20 computer aided design of appropriate derivatives and
analogues can be used.

A further aspect of the invention comprises a method
of monitoring the severity of a disease condition
25 mediated by cellular apoptosis in a cell, tissue or
organism comprising measuring the level of
transcription, expression or activity of a Gas1
protein or a functional equivalent, derivative or
bioprecursor thereof in said cell or tissue or
30 organism.

The present inventors have also now, advantageously,
identified the sequence of the rat Gas1 polypeptide
and which has never before been fully characterised.
35 Accordingly, a further aspect of the present invention
comprises a nucleic acid molecule encoding a rat Gas1
protein or a functional equivalent, derivative or

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bioprecursor thereof, comprising an amino acid sequence according to sequence ID No. 2. Preferably, the nucleic acid molecule is a DNA molecule and even more preferably a cDNA molecule, which in a preferred embodiment comprises the sequence of nucleotides according Sequence ID No. 1. Alternatively, the invention provides in a further aspect, a nucleic acid molecule encoding a protein capable of inducing apoptosis in a cell, comprising an amino acid according to Sequence ID No. 4 or a nucleic acid molecule which is complementary thereto.

The invention also provides an antisense molecule capable of hybridising to a nucleic acid molecule according to the invention under conditions of high stringency.

Stringency of hybridisation as defined herein refers to conditions under which polynucleic acids are stable. The stability of hybrids is reflected in the melting temperature (T_m) of the hybrids. T_m can be approximated by the formula:

$$81.5^{\circ}\text{C} - 16.6 (\log_{10}[\text{Na}^+] + 0.41 (\% \text{G\&C}) - 600/1$$

wherein 1 is the length of the hybrids in nucleotides. T_m decreases approximately by 1-1.5°C with every 1% decrease in sequence homology.

Preferably, the antisense molecule comprises the sequence of nucleotides according to Sequence ID No. 5.

Advantageously, the nucleic acid molecule according to the invention may be used to express the Gas1 protein according to the invention, in a host cell or the like using an appropriate expression vector.

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An expression vector according to the invention includes vectors capable of expressing DNA operatively linked to regulatory sequences, such as promoter regions, that are capable of effecting expression of such DNA fragments.

Regulatory elements required for expression include promoter sequences to bind RNA polymerase and transcription initiation sequences for ribosome binding. For example, a bacterial expression vector may include a promoter such as the lac promoter and for transcription initiation the Shine-Dalgarno sequence and the start codon AUG. Similarly, a eukaryotic expression vector may include a heterologous or homologous promoter for RNA polymerase II, a downstream polyadenylation signal, the start codon AUG, and a termination codon for detachment of the ribosome. Such vectors may be obtained commercially or assembled from the sequences described by methods well known in the art. Thus, an expression vector refers to a recombinant DNA or RNA construct, such as a plasmid, a phage, recombinant virus or other vector that upon introduction into an appropriate host cell result in expression of the DNA or RNA fragments. Appropriate expression vectors are well known to those skilled in the art and include those that are replicable in eukaryotic cells and/or prokaryotic cells and those that remain episomal or those which integrate into the host cell genome.

The antisense molecule capable of hybridising to the nucleic acid according to the invention may be used as a probe or as a medicament or alternatively in a pharmaceutical composition, by preventing expression of a Gas1 protein. Advantageously, the antisense molecule according to the invention may be used as a drug, or in the preparation of a drug for the

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treatment of the previously described pathological processes. The expression vector including said antisense molecule according to the present invention may be used advantageously *in vivo*, such as in gene therapy (Matteucci & Wagner, Nature 384, Supp 7:20-22 (1996); Whitesell et al. Proc. Natl. Acad. Sci. USA 90:4665-4669 (1993); Wahlestedt, C. Trends Pharmacol. Sci. 15:42-46 (1994)).

10 Nucleic acid molecules according to the invention may be inserted into the vectors described in an antisense orientation in order to provide for the production of antisense RNA. Antisense RNA or other antisense nucleic acids may be produced by synthetic means.

15 A further aspect of the invention comprises the host cell transformed, transfected or infected with the expression vector according to the invention, which cell preferably comprises a eukaryotic cell and more preferably a mammalian cell.

20 Incorporation of cloned DNA into a suitable expression vector for subsequent transformation of a cell and subsequent selection of the transformed cells is well known to those skilled in the art as provided in
25 Sambrook et al (1989) Molecular Cloning, A Laboratory manual, Cold Spring Harbour Laboratory Press.

30 A further aspect of the present invention comprises a nucleic acid molecule having at least 15 nucleotides of the nucleic acid molecule according to the invention and preferably from 15 to 50 nucleotides.

35 These sequences may, advantageously be used as probes or primers to initiate replication or the like. Such nucleic acid molecules may be produced according to techniques well known in the art, such as by

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recombinant or synthetic means. They may also be used in diagnostic kits or devices or the like for detecting for the presence of a nucleic acid according to the invention. These tests generally comprise contacting the probe with a sample under hybridising conditions and detecting for the presence of any duplex formation between the probe and any nucleic acid in the sample.

According to the present invention these probes may be anchored to a solid support. Preferably, they are present on an array so that multiple probes can simultaneously hybridize to a single biological sample. The probes can be spotted onto the array or synthesised *in situ* on the array. (See Lockhart et al., Nature Biotechnology, vol. 14, December 1996 "Expression monitoring by hybridisation into high density oligonucleotide arrays". A single array can contain more than 100, 500 or even 1,000 different probes in discrete locations.

Nucleic acid molecules according to the invention may also be produced using such recombinant or synthetic means, such as, for example, using PCR cloning mechanisms which generally involve making a pair of primers, which may be from approximately 10 to 50 nucleotides to a region of the gene which is desired to be cloned, bringing the primers into contact with mRNA, cDNA, or genomic DNA from a human cell, performing a polymerase chain reaction under conditions which bring about amplification of the desired region, isolating the amplified region or fragment and recovering the amplified DNA. Generally, such techniques as defined herein are well known in the art, such as described in Sambrook et al (Molecular Cloning: a Laboratory Manual, 1989).

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The nucleic acids or oligonucleotides according to the invention may carry a revealing label. Suitable labels include radioisotopes such as ^{32}P or ^{35}S , enzyme labels or other protein labels such as biotin or fluorescent markers. Such labels may be added to the nucleic acids or oligonucleotides of the invention and may be detected using known techniques *per se*.

Further provided by the present invention is a transgenic cell, tissue or organism comprising a transgene capable of expressing the rat Gas1 protein according to the invention. The term "transgene capable of expression" as used herein means any suitable nucleic acid sequence which leads to expression of a Gas1 protein having the same function and/or activity of a rat Gas1 protein according to the invention. The transgene may include, for example, genomic nucleic acid or synthetic nucleic acid including cDNA, integrated into the chromosome or in an extrachromosomal state.

Preferably, the transgene comprises a vector according to the invention, which vector includes a nucleic acid molecule encoding said rat Gas1 protein, or a functional fragment of said nucleic acid molecule. A "functional fragment" of said nucleic acid should be taken to mean a fragment of the gene or cDNA encoding said rat Gas1 or a functional equivalent thereof, which fragment is capable of being expressed to produce a functional rat Gas1 protein according to the invention.

In accordance with the present invention, a defined nucleic acid includes not only the identical nucleic acid but also any minor base variations including in particular, substitutions in bases which result in a synonymous codon (a different codon specifying the

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same amino acid residue) due to the degenerate code in conservative amino acid substitutions. The term "nucleic acid molecule" also includes the complementary sequence to any single stranded sequence given regarding base variations.

The invention provides for the rat Gas1 proteins themselves, encoded by the nucleic acid molecules according to the invention. Preferably, the Gas1 protein comprises the sequence of amino acids according to the sequence of amino acids of Sequence ID No. 2. In a further aspect of the invention comprises a protein capable of inducing apoptosis in a cell comprising an amino acid sequence according to Sequence ID No. 4 or a functional equivalent, derivative or bioprecursor thereof.

A "functional equivalent" as defined herein should be taken to mean a rat Gas1 protein that exhibits all of the growth properties and functionality associated with rat Gas1 protein. A "derivative" as defined herein is intended to include a polypeptide in which certain amino acids have been altered or deleted or replaced with other amino acids and which polypeptide retains the biological activity of Gas1 according to the invention and/or which polypeptide can react with antibodies raised using Gas1 according to the invention as the challenging antigen.

Encompassed within the scope of the present invention are hybrid and modified forms of rat Gas1, including fusion proteins and fragments. The hybrid and modified forms include, for example, when certain amino acids have been subjected to some modification or replacement, such as for example, by point mutation yet which modifications still result in a protein which retains the biological activity of Gas1,

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according to the invention. Specific nucleic acid sequences can be altered by those of skill in the art to produce a protein exhibiting the same or substantially properties to Gas1 of the invention.

5

A defined protein, polypeptide or amino acid sequence according to the invention includes not only the identical amino acid sequence but isomers thereof in addition to minor amino acid variations from the natural amino acid sequence including conservative amino acid replacements (a replacement by an amino acid that is related in its side chains). Also included are amino acid sequences which vary from the natural amino acid but result in a polypeptide which is immunologically identical or similar to the polypeptide encoded by the naturally occurring sequence.

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Proteins or polypeptides according to the invention further include variants of such sequences, including naturally variants which are substantially homologous to said proteins or polypeptides. In this context, substantial homology is regarded as a sequence which has at least 70%, and preferably 80%, 90% or 95% amino acid homology with the proteins or polypeptides encoded by the nucleic acid molecules according to the invention.

30

35

Substantial homology should be taken to mean that the nucleotide and amino acid sequences of the Gas1 of the invention display a certain degree of sequence identity. Preferably they share an identity of at least 30%, preferably 40%, more preferably 50%, still more preferably 60%, most preferably 70%, and particularly an identity of at least 80%, preferably more than 90% and still more preferably more than 95 % is desired with respect to the nucleotide or amino

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acid sequences depicted in Seq. ID Nos. 1 to 4,
respectively. A preferred method for determining the
best overall match between a query sequence (a
sequence of the present invention) and a subject
5 sequence, also referred to as a global sequence
alignment, can be determined using, for example, the
FASTDB computer program based on the algorithm of
Brutlag et al. (Comp. App. Biosci. 6 (1990), 237-245.)
In a sequence alignment the query and subject
10 sequences are both DNA sequences. An RNA sequence can
be compared by converting U's to T's. The result of
said global sequence alignment is in percent identity.
Further programs that can be used in order to
determine homology/identity are described below and in
15 the examples. The sequences that are homologous to the
sequences described above are, for example, variations
of said sequences which represent modifications having
the same biological function, in particular encoding
proteins with the same or substantially the same
20 receptor specificity, i.e. binding specificity. They
may be naturally occurring variations, such as
sequences from other mammals, or mutations. These
mutations may occur naturally or may be obtained by
mutagenesis techniques. The allelic variations may be
25 naturally occurring allelic variants as well as
synthetically produced or genetically engineered
variants.

Antibodies to the Gas1 protein according to the
30 invention may advantageously be prepared by techniques
which are well known to those of skill in the art.

The therapeutic or pharmaceutical compositions of the
present invention can be administered by any suitable
35 route known in the art including for example
intravenous, subcutaneous, intramuscular, transdermal,
intrathecal or intracerebral or administration to

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cells in ex vivo treatment protocols. Administration can be either rapid as by injection or over a period of time as by slow infusion or administration of slow release formulation. For treating tissues in the central nervous system, administration can be by injection or infusion into the cerebrospinal fluid (CSF).

Gas1 protein, the antisense molecules or indeed the compounds identified as enhancers or inhibitors of activity or expression of Gas1 or the otherwise lethal proteins may be used in the form of a pharmaceutical composition, which may be prepared according to procedures well known in the art. Preferred compositions include a pharmaceutically acceptable vehicle or diluent or excipient, such as for example, a physiological saline solution. Other pharmaceutically acceptable carriers including other non-toxic salts, sterile water or the like may also be used. A suitable buffer may also be present allowing the compositions to be lyophilized and stored in sterile conditions prior to reconstitution by the addition of sterile water for subsequent administration. Incorporation of the aforementioned compounds or antisense molecules, for example, into a solid or semi-solid biologically compatible matrix may be carried out which can be implanted into tissues requiring treatment.

The carrier can also contain other pharmaceutically acceptable excipients for modifying other conditions such as pH, osmolarity, viscosity, sterility, lipophilicity, solubility or the like. Pharmaceutically acceptable excipients which permit sustained or delayed release following administration may also be included.

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The compounds identified in accordance with the method of the invention may be administered orally. In this embodiment they may be encapsulated and combined with suitable carriers in solid dosage forms which would be well known to those skilled in the art.

As would be well known to those of skill in the art, the specific dosage regime may be calculated according to the body surface area of the patient or the volume of body space to be occupied, dependent upon the particular route of administration to be used. The amount of the composition actually administered will, however, be determined by a medical practitioner, based on the circumstances pertaining to the disorder to be treated, such as the severity of the symptoms, the composition to be administered, the age, weight, and response of the individual patient and the chosen route of administration.

The invention may be more clearly understood from the following examples and accompanying figures wherein:

Figure 1 is a graphic representation of induction of cell death in hippocampal neurons by transient overexpression of Gas1. Quantitative analysis of the effect of rat Gas1 overexpression is represented as % survival 24 hours after transfection of the different expression vectors and the combination of expression vectors, that is showed under each column. Values are referred to 100% survival which corresponds to transfection with empty vector pcDNA3 as indicated in the first column. Overexpression of Gas1 resulted in a reduction of neuronal survival to 30% (1.5 μ g of Gas1, rg1) or 50% (0.75 μ g of Gas1, rg1*). Overexpression of the C-terminal truncated form of Gas1 (Δ C) did not have a significant effect on cell survival at 24 hr. Cotransfection of Gas1 and Bcl-2 resulted in the total

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protection of the neurons (rgl*+Bcl-2*). While overexpression of Bcl-2 alone (1.5 μ g) or the complementary form of Gas1 (AS, 1.5 μ g) did not result in survival values different from the control. Results are expressed as mean \pm SEM. Statistical analysis was performed by Student's t-test. *** denotes $p > 0.001$, and other treatment groups were statistically non-significant ($p < 0.5$).

Figure 2 is a graphic representation of the effects of protection against cell death by an antisense Gas1 oligonucleotide and by complementary Gas1 RNA.

A.- Antisense Gas1 (25 μ M Ag1) blocks NMDA-induced neuronal death in cortico-hippocampal cultures. The viability of mature cortico-hippocampal cultures was analyzed 24 hr after 500 mM NMDA exposure.

Pretreatment with the different antisense oligonucleotides at the indicated concentrations was done 24 hr before the exposure to NMDA. Results are expressed as mean \pm SEM. Statistical analysis was performed by Student's t-test. *** denotes $p > 0.001$, and other treatment groups were statistically non-significant ($p < 0.5$).

B.- Antisense Gas1 (25 μ M Ag1) prevents the reduction in survival induced by 200nM staurosporine in NB69 wild type cells (NB69wt). Columns 1 and 2. Moreover, stable expression of complementary Gas1 RNA in NB69-Gas1 cells protects from staurosporine-induced neuronal death as compared to NB69 mock cells stably transfected with the empty vector. Columns 3 and 4. The viability was analyzed 24 hr after exposure to staurosporine (200nM) in all the cases. Results are expressed as mean \pm SEM. Statistical analysis was performed by Student's t-test. *** denotes $p > 0.001$, and other treatment groups were statistically non-significant ($p < 0.5$).

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Figure 3 is an illustration showing increase in LDH levels in the culture media at different times after doxycycline removal from indicated clones.

5 Figure 4 is an illustration of the results obtained from a Western blot analysis showing the induction of Gas-1 immunoreactivity after doxycycline removal from indicated clones.

10 Figure 5 is an illustration of the results obtained from staining with Annexin-V Fluos (green) before (A) and 24 h. after (B) removal of doxycycline in cultures of NB69-MC3 cells. Propidium iodine staining could be observed at later times (48 h. or more) after
15 doxycycline removal © and D).

Figure 6 is a diagrammatic representation of results showing reduced LDH release to the culture media after doxycycline removal in C3 cells transiently
20 transfected with IAP.

Figure 7 is a diagrammatic representation of results showing increase in LDH levels in the culture media and reduced beta-galactosidase activity after
25 transient transfection of wtGas1 or mutants $\Delta 1$ to $\Delta 3$.

Figure 8 is an illustration of results showing fragmentation of chromosomal DNA in NB69 cells treated with 200 nM staurosporine is prevented by stable
30 overexpression of antisenseGas1. Standard ladder DNA is shown in lane 1 (M).

Figure 9 is a diagrammatic representation of the increase in LDH release and the reduction in beta-galactoside activity after overexpression of human
35 mGluR1 in NB69 cells is blocked by cotransfection of antisense Gas1.

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Example 1.- Cloning rat Gas1

50,000 primary phages from a cDNA library prepared from cortico-hippocampal primary cultures 6 hr after a brief (5 min.) exposure to 500 mM of NMDA were differentially screened. Primary cortico-hippocampal cultures were prepared from E17 rat fetuses essentially as described (Choi, 1987). Neuronal cultures were used for the experiments after twelve days in culture. To prepare the libraries, poly-A+ mRNA was prepared from cortico-hippocampal control or NMDA treated cultures using the microFastTrack kit from Invitrogen. Oligo-dT primed cDNA was cloned in 1ZAP (Stratagene) following manufacturer's instructions. Fifty thousand phages were differentially screened, using ³²P-labeled cRNA probes derived from poly-A+ RNA isolated from control cultures (- probe), or from cultures 6 hr after the NMDA treatment (+ probe) and the use of the AMV reverse transcriptase (25 U, BRL). For differential screening of the library prepared from treated cultures, each pair of nitrocellulose filters upon which phages have been transferred are subjected to probe (+) or probe (-) hybridization for 20 hours at 42° C in a hybridization buffer that contains 50% formamide, 10% dextrane sulfate, 4xSSC, 0.1% SDS, Tris-HCl 10 mM, pH 7.4, 1xdenhardt's, 50 (g/ml) and salmon sperm and the corresponding probe 106 dpm/ml. Then the filters are washed for 30 min. at room temperature in 2xSSC, 0.1% SDS, twice for 30 min. At 42° C in 2xSSC, 0.1% SDS and twice for 30 min. At 55° C in 0.2xSSC, 0.1% SDS. After autoradiographic exposure for five days, the hybridization signals obtained for each pair of filters hybridized with probe (+) or probe (-) are compared, looking for the differential presence of hybridization signal in filters (+) in relation to (-). In this way a 714 bp

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clone initially named pCHN-414 is obtained, and once sequenced, it showed homology with the untranslated 3' region of the Gas1 human and rat gene. This indicates that the pCHN-414 clone corresponds to the rat Gas1 gene, which is confirmed in the following experiments.

A cDNA fragment containing the complete coding sequence of rat Gas1 was obtained following PCR using rat genomic DNA as template. The 5' primer was derived from the mouse sequence (Del Sal et al., 1992) at position 49-66 (5'-GAATTCGAGAAACGCTCCGAGTTTCG-3'). The sequence of the 3' primer was obtained from the rat clone (5'-GGATCCAGTTTTTAATACAGTTTATACGACGTACCAGG -3') , at a position corresponding to 2449-2483 in the mouse sequence. Flanking EcoRI and BamHI sites in the 5' ends of the oligonucleotides were designated for cloning purposes. The DNA was then amplified by the polymerase chain reaction using Tli DNA polymerase (Promega) for 40 cycles. The timing for each cycle was as follows: 1 min. at 94°C, 1 min. at 60°C and 2 min. at 72°C. The cycling was preceded by 2 min. denaturing period at 94°C and followed by a 7 min. extension at 72°C. A 2.4 kb PCR product was obtained, restricted by EcoRI, cloned into pCDNA3 (Invitrogene) and completely sequenced at both strands using Thermo stable Sequenase and the conditions suggested by the manufacturer (Amersham-Pharmacia).

Example 2.- Gas1 gene transfection induces neuronal death

To assess the effect of the Gas1 protein on neuronal viability, pGas1 was cotransfected together with a β -galactosidase expression vector that served as a marker of transfected neurons using liposomes. For optimal liposome-mediated transfection efficiency these experiments were performed in serum-free

conditions using primary cultures of hippocampal neurons. Culture of hippocampal neurons was performed in chemically defined medium as described (Ohsawa, et al, 1993. Response of embryonic hippocampal neurons in culture to neurotrophin-3, brain-derived neurotrophic factor and basic fibroblast growth factor. *Neurosci.* 57, 67-77.). Hippocampal neurons (3 DIV, 2×10^6 cells per 35 mm dish) received 2 μ g total amount of plasmid DNA containing 0.5 μ g of β -galactosidase expression vector (pCH110, Pharmacia) and 1.5 μ g of pCDNA3 vector (InVitrogene), empty or containing the Gas1 coding sequence in the 5' to 3' orientation (rg1), the Gas1 coding sequence in the complementary orientation (3' to 5', AS) or the truncated Gas1 (Δ C, The C-terminal truncated expression vector for Gas1 contains amino acids 1 to 229 and was prepared from the rg1 plasmid by subcloning of a HindIII/EcoRI fragment into pCDNA3). For the protection by Bcl-2, 0.75 μ g of rg1 and 0.75 μ g of pBcl-2 were used together with 0.5 μ g of pCH110. A ratio of 1:2 DNA: Transfectam (Promega) was used for the preparation of liposomes. Three hours after transfection, the liposome-containing medium was replaced with fresh medium. To assess the number of transfected neurons, the cultures were fixed with 2.5% glutaraldehyde 24 hr after the transfection and X-Gal staining was performed as suggested (Promega). The experiments were repeated 3 to 5 times, performed in quadruplicates and each plate was counted by three independent investigators. X-Gal positive neurons present in 20 fields of each plate were counted and averaged. The average of X-Gal positive neurons in control cultures was taken as 100% survival for each experiment. The transfection efficiency was similar for the different plasmids, for different preparations of a given plasmid and among experiments, as assayed following a similar protocol but 16 hr after transfection, a time point at which cell death has not

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started. The transfection efficiency accounted for approximately 1 % of the total cell population, and showed little variation between the different experimental groups and between experiments.

5 At 24 hr after transfection, the total number of X-Gal positive neurons in cultures transfected with rg1 was dramatically reduced. The survival after Gas1 overexpression was decreased in a concentration-dependent manner to 30% and 50% when transfected with 10 1.5 and 0.75 μ g of pGas1, respectively (Fig. 1). In contrast, the overexpression of the complementary strand of Gas1 (AS), did not affect the number of viable X-Gal positive cells at 24 hr after transfection (Fig. 1). These results associate the 15 overexpression of Gas1 with neuronal death and indicate that high levels of Gas1 protein are sufficient to trigger a death process that is accompanied by profound alterations in morphology.

20 **Example 3.- Blocking of translation of the Gas1 protein by Gas1 antisense oligonucleotide or by Gas1 complementary chain overexpression protects against excitotoxic death or death induced by staurosporine.**

25 NMDA-induced excitotoxicity was analyzed in cortico-hippocampal cultures maintained for 12-14 DIV similar to those already described in Example 1. To apply the excitotoxic insult, the cultures were rinsed twice with Locke's solution without Mg²⁺ and exposed to 500 30 mM NMDA (Sigma) for 5 min. in the same Locke's solution or to Locke's solution alone as control. NMDA was washed out, the Mg²⁺ concentration was restored by 2 changes of Locke's +Mg²⁺ solution, and the original medium was replaced. Phase-bright bipolar cells, taken 35 to represent living neurons, were counted 24 hr after the NMDA exposure. Alternatively, the vital staining method (Jones and Senft, 1985 An improved method to

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determine cell viability by simultaneous staining with fluorescein diacetate-propidium iodide. J Histochem Cytochem 33:77-79) was used to assess the extent of neuronal death and the protection by antisense oligonucleotide treatments. Similar results were obtained using either method. The vital staining method was used to prepare Fig. 2a.

A 15-mer Gas1 antisense oligonucleotide 5'-TCCTCATCCATCCAT-3', (AG1) spanning the start site of translation (underlined) was used to specifically block Gas1 translation. As a negative control a 15-mer oligonucleotide with nucleotide substitutions 5'-TCCTCATCGATGGTA-3' (AG1mut) was used and the Gas1-unrelated antisense oligonucleotides for cyclin D1: 5'-GAGCTGGTGTTCAT -3' (Matsushime et al., 1991 Colony-stimulating factor 1 regulates novel cyclins during the G1 phase of the cell cycle Cell 65:701-713) and for zif268: 5'-GTAGTTGTCCATGGT -3' (Milbrandt, 1987 A nerve growth factor-induced gene encodes a possible transcriptional regulatory factor. Science 238:797-799). All oligonucleotides were protected at each end by two phosphorothioate groups. The oligonucleotides were added to the culture medium 24 hr before the induction of neuronal death by NMDA, at the concentrations indicated in the figures. Experiments were done in triplicate and repeated as least five times.

Staurosporine-induced neuronal death was assayed in human neuroblastoma NB69 cells cultured in DMEM/HAM F12 medium supplemented with 10% fetal calf serum, 2 mM glutamine and 50 µg/ml gentamycin. Wild-type or stably transfected NB69 cells (2 x 10⁴ cells/35 mm dish) were exposed to 100 nM staurosporine (RBI). Phase-bright cells without signs of membrane or neurite degeneration were counted 24 hr later. Experiments were done in triplicate and repeated as

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least five times. Stably transfected NB69 cells that overexpress Gas1 in the antisense orientation (NB69-Gas1) were prepared by the calcium phosphate precipitation method followed by two weeks of geneticin selection. Control cells were prepared in parallel by transfection with pCDNA3 vector alone (NB69-vector). Expression of the constructs in the cell clones were analyzed by northern blot analysis, and the clones having the highest expression were chosen for the experiments. Stable transfectants were kept in the presence of 0.3 mg/ml of geneticin, which was removed before plating for the experiments.

Neuron death by overstimulation of excitatory amino acid receptors is a well characterized phenomenon that receives the generic denomination of excitotoxicity. Excitotoxicity is a complex process that involves significant changes in the cell cycle gene expression.

The detection of various of these genes was carried out in the course of differential screening of primary culture rat cortico-hippocampal neuron library at an excitotoxic concentration (500 mM) of N-methyl D-aspartic-NMDA acid (Choi, 1987, Ionic dependence of glutamate neurotoxicity in cortical cell culture. J. Neurosci 7:369-379). Cloning and sequence determination of one of them identified it as part of the rat homologue of the Gas1 gene described above in humans. Subsequently, the complete rat Gas1 gene was cloned and sequenced, as referred to below whose nucleotide sequence is attached as SEQ ID 1, obtaining from it the amino acid sequence of the rat Gas1 protein whose sequence is attached as SEQ ID 2.

Subsequently, the increase of 8 to 10 times the Gas1 mRNA expression after 6 hours of administering a pulse of NMDA in primary cortico-hippocampal cell cultures

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in contrast to control cells was confirmed by Northern blot. On the other hand, Gas1 mRNA induction has not been observed in pure primary culture of hippocampal astroglia after exposure to NMDA or glutamate (data not shown), suggesting that Gas1 overexpression occurs in neurons as part of the gene response during NMDA induced neuronal degeneration.

Hence, Gas1 expression in experimental models where neuron death was induced by means of different harmful stimuli has been evaluated. First of all, intraperitoneal administration of kainic acid was used, an experimental model that has demonstrated that it produces acute and delayed neuron death in different cerebral areas that include the hippocampus, olfactory cortex, thalamus and amygdala ((Schwob et al, Widespread patterns of neuronal damage following systemic or intracerebral injections of kainic acid: a histological study. *Neurosci* 5:991-1014; 1980; Sperk et al., Kainic acid-induced seizures: neurochemical and histopathological changes. *Neurosci* 10:1301-1315;1983). Northern blot analysis demonstrated that Gas1 expression in control rat brain (without administering kainic acid) was very low in accordance with previous data (Del Sat et al., 1994). However, strong induction of Gas1 expression in hippocampus and olfactory cortex after intraperitoneal administration of 10 mg/kg of kainic acid was observed. Already detectable induced Gas1 mRNA levels after kainic acid appear 6 hours after the beginning of treatment and were maintained for at least 10 days. In order to reveal Gas1 induction and to confirm neurone localization thereof in situ hybridization after administering kainic acid was performed. A RNA specific for Gas1 monocatenary probe revealed, 24 hours after administration of kainic acid, signs of intense hybridization in isolated neurons, being evident in the pyramidal layer of the CA1 area of the

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hippocampus. Similar results were observed after provoking an experimental ischemic process in gerbil brain by transient occlusion of both carotids, giving rise to rapid degeneration of a numerous population of pyramidal neurons of the CA1 layer of the hippocampus. It was observed that this massive neuron death was preceded by a significant rise of Gas1 mRNA and Gas1 protein levels (evaluated by immunocytochemistry) in those neurons. Likewise, it was also observed that neuron loss in all the cerebral cortex that is produced in rats in the perinatal period after exposure to ionizing radiation is preceded by high induction of Gas1 mRNA. All these results suggest that Gas1 is involved in regulation of neuron death by excitotoxicity.

In a subsequent step to show a direct relationship between Gas1 gene expression and neuron death, hippocampal neurons were transfected with the pcDNA3 eukaryotic expression vector containing the Gas1 (rgl) gene. The experiments were carried out in primary cultures of rat fetus hippocampal neurons that were transfected with different amounts of the Gas1 expression vector, evaluating a posteriori the survival of these cells (Fig. 1). Hence, it was observed that transfection of cells with 1.5 μ g of rgl construct caused a significant reduction of survival, obtaining levels of survival lower than 40%, with regard to the control group (transfected with the empty expression vector), or with regard to the group of cells transfected with the Gas1 (SEQ ID 3) complementary or antisense sequence (Antisense(AS) column, see figure 1). The effect depends on the amount of Gas1 construct transfected and thus when transfection took place with half the amount (rgl*) the reduction of survival was more limited although still significant (Fig. 1).

On the other hand, this reduction of survival of neurons transfected with Gas1 (rgl* column) is completely blocked by coexpression in these same neurons with the Bcl-2 protein (rgl*+Bcl-2 column), Bcl-2 being the antiapoptotic protein prototype (Fig. 1). On the other hand, these experiments with rat Gas1 were repeated in NB69 human cells and in NIH3T3 rat fibroblasts, obtaining similar results. Besides, NB69 human neuroblastoma and 3T3 rat fibroblast cells were transfected with human Gas1, observing cell death of the NB69 and 3T3 cells.

Initially, Gas1 expression has been related to the G0 phase of cell cycle arrest in rat fibroblasts deprived of serum (Del Sal et al., 1992). However, the present invention shows that Gas1 overexpression in cortico-hippocampal neurons induces neuron death, acting as a protein with apoptosis inductive activity.

Up to now it had been described that Gas1 protein was a cell membrane protein and as of its amino acid sequence the presence of two transmembrane segments had been proposed and a hypothetical RGD domain that may interact with integrin, the only domain described that relates its structure to its arrest function of the cell cycle (Schneider et al.). The present inventors have succeeded in overexpressing the Gas1 protein by means of a prokaryotic vector (pTrcHis) in bacteria for subsequent purification in sufficient amounts to carry out protein studies. Hence, it was observed in experiments of artificial bilayers that the purified Gas1 protein is capable of inserting in and forming cationic channels and that said capacity may be linked to neuron death induction. Aside from the already described location of this protein in the cell membrane we have located the presence of Gas1 in the perinuclear and mitochondrial membrane. It must

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be pointed out that Bcl-2 and Bax, proteins related to the cell cycle, are also located in the mitochondrial membrane and are capable of forming ionic channels.

This common location of these proteins makes

5 compatible a possible functional interaction of Gas1 with Bax type proapoptotic proteins and Bcl-2 and Bcl XL antiapoptotic proteins. The present inventors have also been able to demonstrate, by mutational analysis of the RGD domain, that this is not implied in neuron
10 apoptotic activity of the Gas1 protein. On the contrary, we have delimited the neuron death inductive capacity in an amphipathic domain in the Gas1 terminal carboxyl region, located between the amino acids 174 and 304 of the protein (SEQ ID 4), that does not
15 coincide with any of the previously hypothesized domains.

In order to confirm the relevance of this region of the protein in the lethal capacity of Gas1 hippocampal
20 neurons were transfected with a mutated Gas1 construction in that region (Δ C column, Fig. 1). As seen in Fig. 1, survival of these neurons is similar to that verified in the control group (vector column) and is significantly higher than that of neurons
25 transfected with wild type Gas1 (rg1 and rg1* columns).

Thanks to the exact knowledge of the structure activity relationship in Gas1 polypeptide there is now
30 a rational base to understand the lethal action of Gas1 as well as for the development of molecules capable of blocking the Gas1 polypeptide function or Gas1 gene expression with which to create new therapeutic tools to reduce or eliminate cell death.
35 For this purpose, overexpression of Gas1 protein in human neuroblastoma cells or in rat fetus neurons by means of transfection with liposomes of the Gas1

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eukaryotic expression vector was used. This may be constitutive expression (pcDNA3) in transient expression but it may also be an inducible expression (pIND) that permits preparation of stable lines that will express Gas1 after induction of the expression vector for example with muristerone in the case of pIND. To evaluate the phenomenon of death one can resort to morphological criteria by means of transfection with b-galactosidase or by means of GFP (green fluorescent protein) or to biochemical criteria of degradation, detection of nucleic acids or release of dehydrogenase lactate into the medium, or any other indication of cellular lysis. The specific application of this invention is its use as a screening system of molecule collections in the search for products active in blocking Gas1 polypeptide function or Gas1 gene expression and therefore, providing a protective action against cell death. These screening systems form part of the present invention.

Transgenic models provide a useful model for assaying and testing drugs for their effectiveness and safety in the treatment of the above described diseases. Also, by means of the use of specific tissue promoters or cellular phenotype promoters Gas1 expression can be achieved in target tissues and cells for a better and more specific testing. An advantage of the invention is that potential Gas1 inhibitors and/or blocking agents can be readily tested in an *in vivo* model that closely mimics a human by the use of a rat gene for Gas1.

Furthermore, a specific Gas1 antisense oligonucleotide (SEQ ID 5) was verified as being able to block the translation of Gas1 protein and this involves a total blocking of the NMDA induced neuron death phenomenon in primary neuron cultures (survival practically of

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100%, 25mM Ag1 column in Fig. 2a) or by staurosporin induced death (200nM) (Fig. 2b).

5 Upon observing that the stable expression of high levels of Gas1 complementary messenger in the NB69-Gas1 cell lines makes these cells more resistant to external harmful stimuli, it was possible to use these cells for forced expression of lethal genes that would induce cell death in cells but that would not cause death in these Gas1 expressing protected lines. An example of the possible application of this protected system (NB69-Gas1) is the overexpression of the glutamate type I metabotropic receptor (mGluR-I). This provides a system which permits the study of the pharmacology of mGluR-I and to screen possible agonist or antagonist molecules of this receptor. The example presented for mGluR-I is only one of the multiple possibilities of using Gas1 inhibition to achieve stable expression systems for genes that are normally detrimental to the survival of a cell. Other lethal proteins to evaluate by means of this expression system comprise, for example, ionotropic glutamate receptors including NMDA-receptors, AMPA receptors, kainate receptors with their various subunits and variants: metabotropic glutamate receptors (including subtype 1 to 8); other excitatory amino acid receptors (e.g. taurine); cytokine receptors; chemokine receptors; mono amine receptors; peptide receptors; enzymes such as kinases, caspases; and any other protein in signal transduction cascade mediating cell death.

35 The present inventors have also developed and produced polyclonal rabbit antibodies and rat hybridomas which can produce Gas1 monoclonal antibodies. These antibodies have been used in the immunocytochemical and Western blot studies that are described in this

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invention for the purpose of evaluating the expression of Gas1 protein. It is to be emphasized that these antibodies may be used for analytical purposes to purify said polypeptide, for in vitro diagnosis or for therapeutic purposes of disorders, especially those that imply cell death and neurodegeneration, where this polypeptide is expressed.

Effect of functional inhibition of Gas1 on neuronal viability.

Preparation of a human Gas1 inducible expression vector using the Tet-Off system. Firstly, an attempt was made to obtain double transfectants RXR-pINDhGas1 for ecdysone-induced expression of hGas-1 in the human neuroblastoma cell line NB69. This approach proved to be difficult and no clones showing a reasonable growth rate could be selected. In most cases, double transfectants presented long neuritic processes corresponding to a phenotype of terminally differentiated neurons, had insignificant incorporation of ³H-thymidine and a notorious accumulation of apoptotic bodies could be observed in the cultures. Attempts to reduce the percentage of serum in the media or to culture under serum-free conditions did not significantly improve the situation. It may be that a very high expression of the RXR receptor in NB69 primary clones NB69-RXR4 and NB69-RXR7 leads to a ligand-independent activation of the pINDhGas1 vector which could be the origin of the problem. Accordingly, NB69-RXR clones expressing low or moderate levels of the RXR receptor were tried. Thus, NB69 cells were cotransfected with RXR and pINDhGas1 and a double selection with G418 and Zeocin was performed. Three weeks after transfection clones could be observed. However, the growth rate of these clones was again very slow and abundant apoptotic

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bodies could be distinguished surrounding and on the top of each clone. In view of these negative results the ecdysone-based approach was abandoned in favour of a different inducible system performed in this case the Tet-Off system from (available from Clontech). Two complete rounds of selection were performed. NB69 cells were initially stably transfected with the TetOff plasmid using G418 for selection. Twelve clones were chosen in each round, 1 to 12 in the first and A to L in the second, for checking of expression levels of the TetOff repressor by transient transfection with reporter TRE-Luc again available from Clontech. After this analysis, clones 3, 8 and C were finally selected for cotransfection with the inducible expression vector TRE-hGas1 (a plasmid including the hGas1 sequence cloned in the pTRE2 vector available from Clontech) and the selection vector TK-hyg (Clontech). Selection was carried out for two weeks using 2 mg/ml of hygromycin in the presence of 1 µg/ml of doxycycline to keep the repressor TetOff bound to the TRE promoter in the TRE-hGas1 construct. Twelve subclones were again chosen from each of the three groups (3, 8 and C) and Gas1-induced cell death was assayed after doxycycline removal using the release of lactate dehydrogenase (LDH) as an index of cell damage (Cytotox kit, Promega). Positive clones were further analyzed for the inducibility of Gas1 after doxycycline removal by western blot using the monoclonal antibody G8F9-1 specific for Gas1. Some of these results are summarized in figures 3 and 4. As a result, five TetOff-inducible clones were finally selected: 8d, 8e, 8f, C2 and C3.

Analysis of the molecular mechanism of Gas1-induced cell death. To assess the cell death mechanism after induction of Gas1 by doxycycline removal we first processed each of the clones using staining for

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annexin-V (Annexin-V Fluos, kit, Roche) as a hallmark of apoptosis and the staining with propidium iodine as an indication of a necrotic mechanism. As shown in figure 5 using clone C3, an intense green fluorescence in the outer membrane was observed soon after doxycycline removal, while at later times some of the cells developed an intense nuclear red fluorescence due to the entrance of the intercalating agent propidium iodine (Fig. 5C and D). These results indicate that the mechanism of death involves a purely apoptotic process, at least at the early stages. Because of this the effect of caspase inhibition on Gas1-induced cell death after doxycycline removal was tested. For that, clones C2 and C3 were transfected with different expression vectors for crmA, IAP and p35, three caspase inhibitors with distinct specificity for different caspases. In these experiments it was found that overexpression of IAP, a caspase inhibitor of non-viral origin, selectively blocked Gas1-induced cell death after doxycycline removal (Fig. 6). This result indicates that inhibition of the pro-caspase 9 or the effector caspases 3, 6 and 7 are involved in the death process triggered by Gas1. Further studies using more selective caspase inhibitors as well as specific substrates for the different caspases are needed to identify the caspase directly responsible of the death after Gas-1 induction.

Molecular analysis of death-related domains in Gas1.

Domain analysis of the amphipathic α -helix region of Gas1. As a result of the frame-shift strategy it was possible to delineate a domain encompassing amino acids 174 to 279 in rat Gas1 involved both in the channel activity as well as in the death-inducing properties of Gas1. To more precisely map the specific

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residues responsible for these two biological activities deletion of discrete fragments of Gas1 within the 174-279 region was performed. Three deletion mutants have, so far, been prepared and analyzed Gas1 $\Delta 1$ to $\Delta 3$. Transient cotransfection experiments of each construct with an expression vector for lacZ was performed in NB69 cells and release of LDH and beta-galactosidase activity was measured as an index of cell death and viability, respectively. In all cases, a lethal effect similar to wtGas1 was observed. The results of a representative experiment are shown in figure 7.

Effect of knockout of Gas1 on neuronal viability.

Stable expression of antisense Gas1 RNA in NB69 cells: effects on survival. To develop a cellular model highly resistant to the overexpression of potentially toxic proteins i.e. metabotropic glutamate receptors, we selected NB69 clones stably transfected with an expression vector for Gas1 cloned in the antisense orientation. The initial screening of positive clones was performed by Northern blot. The clone showing higher levels of antiGas1 mRNA was then tested for its resistance to neuronal death induced by staurosporine. In previous it was observed that staurosporine-induced neuronal death involves the induction of endogenous Gas1 and could be prevented by administration to the culture media of the antisense oligonucleotides described herein specific for the amino-terminal of Gas1. As shown in figure 8, the DNA laddering associated with the apoptosis induced by staurosporine was not observed in cells stably transfected with antisense Gas1.

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Stable expression of human mGluR1 in NB69 cells:

protection by antisense Gas1. The suitability of our NB69-ASGas1 cells to support the stable overexpression of potentially lethal proteins was then tested. In pilot experiments, transient transfections in NB69 cells with an expression vector for the human metabotropic GluR1 was performed and the effects on viability after cotransfection with antisenseGas1 expression vector were compared. The results were again evaluated using the two independent tests of cell death; release of lactate dehydrogenase to the culture media at different times and the survival of transfected cells 48 hours after transfection using the expression of LacZ as an interval reporter. The results obtained from a typical experiment performed in triplicate are shown in Figure 9.

Version to Show Changes Made

The specification has been amended to add the following paragraph after the title of the application:

--This application is a national stage application of PCT/EP00/08182 filed August 21, 2000 entitled "Gas 1 Polypeptides" which claims priority from EP 99306702.4 entitled "Gas1 Polypeptides" filed August 24, 1999, the contents of these applications is incorporated by referenced into this application.--

The claims have been amended as follows:

1. (Amended) A method of inhibiting the lethal effect of expressing an otherwise lethal protein in a cell, said method comprising the steps of:

- (a) providing a cell, tissue or organism having (i) a nucleotide sequence encoding a Gas1 protein, or a functional equivalent, derivative or bioprecursor thereof, which is capable of inducing apoptosis in said cell and (ii) a further nucleotide sequence encoding a protein which is otherwise lethal to said cell in itself or in response to a lethal stimulus in the presence of Gas1;
- (b) inhibiting function and/or expression of said Gas1 protein or functional equivalent, derivative or bioprecursor thereof; and
- (c) expressing said sequence encoding said otherwise lethal protein

wherein said protein normally induces the expression or activates either Gas1 protein or a protein in the signal transduction pathway of which Gas1 is a component.

2. (Amended) A method of identifying compounds which inhibit or enhance expression or activity of proteins which are lethal to a cell, tissue or organism said method comprising the steps of:

- (a) providing a cell, tissue or organism comprising a nucleotide sequence encoding a Gas1 protein or a functional equivalent, derivative or bioprecursor thereof, which is capable of inducing apoptosis in said cell, and ii) a further sequence encoding a protein which is otherwise lethal to said cell in

itself or in response to a lethal stimulus in the presence of Gas1;

- (b) inhibiting function and/or expression of said Gas1 protein or functional equivalent, derivative or bioprecursor thereof or a protein in the apoptotic pathway of which Gas1 is a component;
- (c) expressing said sequence encoding said otherwise lethal protein;
- (d) contacting said cell with a compound to be tested; and
- (d) monitoring the effect of said compound on said otherwise lethal protein compared to an identical cell which has not been contacted with said compound wherein said proteins normally induce the expression or activate either the Gas1 protein or a protein in the signal transduction pathway of which Gas1 is a component.

3. (Amended) A method according to claim [1 or] 2 wherein said expression or activity of Gas1 protein is inhibited by providing a nucleic acid molecule in said cell which is capable of hybridising to mRNA corresponding to Gas1 DNA to prevent expression thereof.

4. (Amended) A method according to claim [1 or] 2 wherein said expression or activity of said Gas1 protein is inhibited by inhibiting the expression or activity of a protein in the pathway of which Gas1 is a component.

5. (Amended) A method according to [any of] claim[s] [1 to 4] 2 wherein said cell is induced to express said Gas1 protein by contacting said cell with a stimulus that increases intracellular calcium levels in said cell.

7. (Amended) A method according to [any of] claim[s] [1 to 6] 2 wherein said further sequence encoding said otherwise lethal protein is expressed by providing it on a suitable expression vector.

8. (Amended) A method according to [any of] claim[s] [1 to 7] 2 wherein said lethal protein is a highly expressed recombinant protein.

9. (Amended) A method according to [any of] claim[s] 1 to 7] 2 wherein said otherwise lethal protein comprises any of a glutamate, NMDA, AMPA or kainate receptor.

11. (Amended) A method according to [any of] claim[s] 3 [to 9] wherein said nucleic acid molecule is provided as an oligonucleotide or as a vector including a nucleotide sequence of said nucleic acid molecule.

14. (Amended) A method according to [any of] claim[s] [1 to 13] 2 wherein said Gas1 protein is of mammalian origin.

16. (Amended) A method according to claim 14 [or 15] wherein said Gas1 protein comprises the amino acid sequence depicted in either of Sequence ID No. 2 or 4 or a functional equivalent, derivative or bioprecursor thereof.

17. (Amended) A compound identifiable as an inhibitor or an enhancer of expression or activity of an otherwise lethal protein according to the method[s of any] of claim[s] 2 [to 15].

22. (Amended) The method of claim 54 [Use according to claim 20 or 21] wherein said cell is associated with a disease condition selected from the group consisting of [comprises any of] a neurological disorder, a cardiovascular disorder, an autoimmune disorder, a neuroendocrine disorder or cancer.

26. (Amended) A nucleic acid molecule according to claim [24 or] 25 which is a DNA sequence.

28. (Amended) A nucleic acid molecule according to claim [24,] 26 [or 27] comprising the sequence of nucleotides according to Sequence ID No. 1.

29. (Amended) An antisense molecule capable of hybridising to the nucleic acid molecule of [any of] claim[s] [24 to 28] 25 under conditions of high stringency.

31. (Amended) A [Gas1] protein encoded by [a] the nucleic acid molecule of Sequence ID NO. 1 or a nucleic acid molecule capable of hybridizing to Sequence ID NO.1 or its complement under conditions of high stringency [according to any of claims 24 to 28].

32. (Amended) A [Gas1] protein comprising the [an] amino acid sequence illustrated in Sequence ID No. 2.

34. (Amended) An expression vector comprising a nucleic acid molecule according to [any of] claim[s] 25 [24 to 28] or a nucleic acid molecule capable of hybridizing to Sequence ID No.1 under conditions of high stringency.

36. (Amended) An expression vector according to claim 34 [or 35] comprising a tissue or cell specific promoter.

37. (Amended) An expression vector according to [any of] claim[s] 34 [to 36] further comprising a sequence encoding a proapoptotic protein.

38. (Amended) An expression vector according to [any of] claim[s] 34 [to 37] which is inducible for expression of [said Gas1 polypeptide] a protein comprising the amino acid sequence of Sequence ID No.2 or [said] a polypeptide capable of inducing apoptosis in a cell.

40. (Amended) A host cell, tissue or organism, transformed, transfected or infected with a vector according to [any of] claim[s] 34 [to 39].

42. (Amended) A method according to claim 41 wherein said cell in step (a) comprises a [cell according to claim 40] host cell, tissue or organism transformed, transfected or infected with a vector comprising a nucleic acid molecule encoding a protein capable of inducing apoptosis in a cell comprising an amino acid sequence according to Sequence ID No. 4 [according to any of claims 24 to 28] or a nucleic acid molecule capable of hybridizing to Sequence ID No.1 under conditions of high stringency.

43. (Amended) A compound identifiable as an inhibitor or an accelerator of cell death according to the method of claim [41 or] 42.

45. (Amended) A pharmaceutical composition comprising any of a nucleic acid molecule encoding a protein according to Sequence ID No. 4 or a nucleic acid molecule capable of

stringent hybridization thereto; [according to any of claims 24 to 28,] an antisense molecule capable of hybridizing to a nucleic acid molecule encoding a protein according to Sequence ID No. 4 under conditions of high stringency; [according to claim 29 or 30,] or a protein encoded by a nucleic acid molecule capable of hybridizing to Sequence ID No. 1 under stringent hybridization conditions [according to any of claims 31 to 33] together with a pharmaceutically acceptable carrier, diluent or excipient therefor.

48. (Amended) The method of [Use according to] claim [47] 22, wherein said neurological disorder is any of, Parkinson's disease, Alzheimer's disease, Huntington's disease, amyotrophic lateral sclerosis, a neurological condition caused by thrombosis or cerebral trauma.

49. (Amended) The method of [Use according to] claim [47] 22, where said cardiovascular disorder is a heart attack.

50. (Amended) The method of [Use according to] claim [47] 22, wherein said autoimmune disorder is multiple sclerosis.

51. (Amended) The method of [Use according to] claim [47] 22, wherein said neuroendocrine disorder is necrosis of the pituitary gland.

52. (Amended) An antibody capable of binding to a protein comprising an amino acid sequence according to Sequence ID NO.2 or Sequence ID No. 4 [according to any of claims 31 to 33].

Claim 54 has been added:

54. A method for decreasing the expression of a protein that is lethal to a cell, wherein the protein normally induces the expression or activation of either the Gas1 protein or a protein in the signal transduction pathway of which Gas1 is a component comprising the step of: contacting the cell with a therapeutically effective dose of a compound identified by (a) providing a cell, tissue or organism comprising a nucleotide sequence encoding a Gas1 protein or a functional equivalent, derivative or bioprecursor thereof, which is capable of inducing apoptosis in said cell, and ii) a further sequence encoding a protein which is otherwise lethal to said cell in itself or in response to a lethal stimulus in the presence of Gas1;
- (b) inhibiting function and/or expression of said Gas1 protein or functional equivalent, derivative or bioprecursor thereof or a protein in the apoptotic pathway of which Gas1 is a component;

- (c) expressing said sequence encoding said otherwise lethal protein;
- (d) contacting said cell with a compound to be tested; and
- (e) monitoring the effect of said compound on said otherwise lethal protein compared to an identical cell which has not been contacted with said compound.

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
1 March 2001 (01.03.2001)

PCT

(10) International Publication Number
WO 01/14549 A1

(51) International Patent Classification⁷: C12N 15/12,
5/10, C07K 14/47, 16/18, C12Q 1/68, G01N 33/50, A61K
31/70, 38/17, A61P 25/00

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(21) International Application Number: PCT/EP00/08182

(22) International Filing Date: 21 August 2000 (21.08.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
99306702.4 24 August 1999 (24.08.1999) EP

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(81) Designated States (national): AE, AG, AL, AM, AT, AU,
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ,
DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR,
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NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM,
TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States (regional): ARIPO patent (GH, GM,
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian
patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European
patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE,
IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG,
CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:

- With international search report.
- Before the expiration of the time limit for amending the
claims and to be republished in the event of receipt of
amendments.

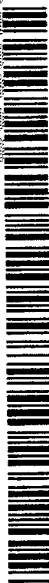
For two-letter codes and other abbreviations, refer to the "Guid-
ance Notes on Codes and Abbreviations" appearing at the begin-
ning of each regular issue of the PCT Gazette.

(54) Title: GAS1 POLYPEPTIDES

(57) Abstract: There is disclosed a method of inhibiting the lethal effect of expressing an otherwise lethal protein in a cell, said method comprising: (a) providing a cell, tissue or organism having (i) a nucleotide sequence encoding a Gas1 protein, or a functional equivalent, derivative or bioprecursor thereof, which is capable of inducing apoptosis in said cell and (ii) a further nucleotide sequence encoding a protein which is otherwise lethal to said cell in itself or in response to a lethal stimulus in the presence of Gas1; (b) inhibiting function and/or expression of said Gas1 protein or functional equivalent, derivative or bioprecursor thereof; and (c) expressing said sequence encoding said otherwise lethal protein.

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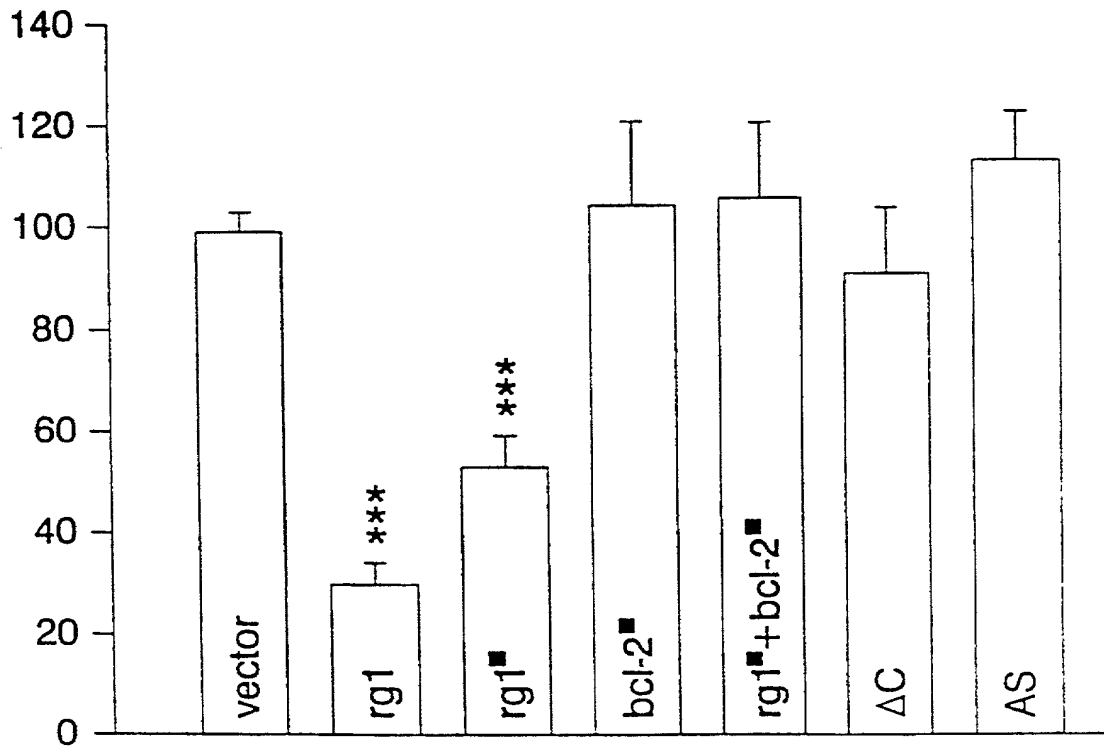


FIG. 1

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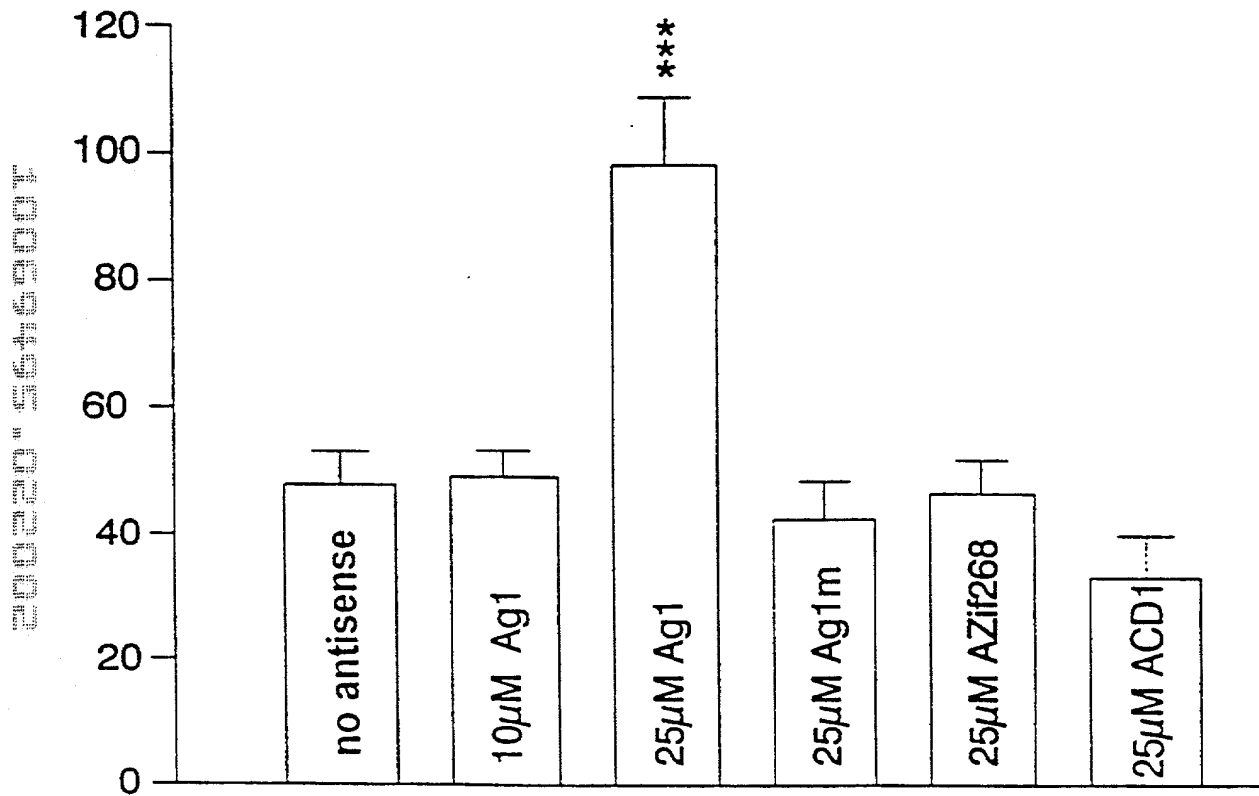


FIG. 2A

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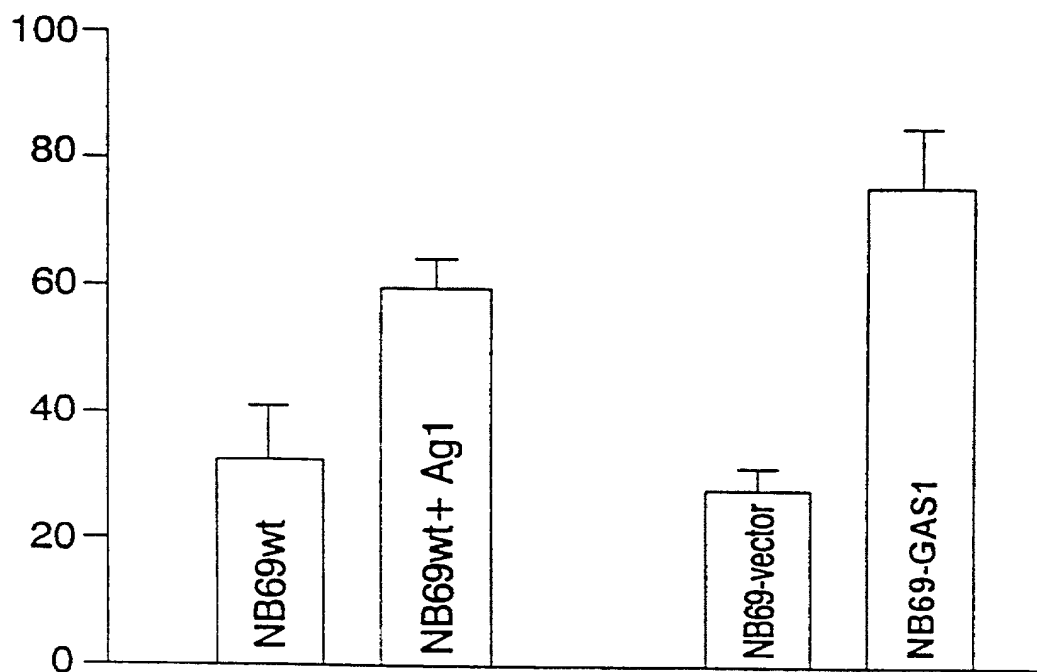
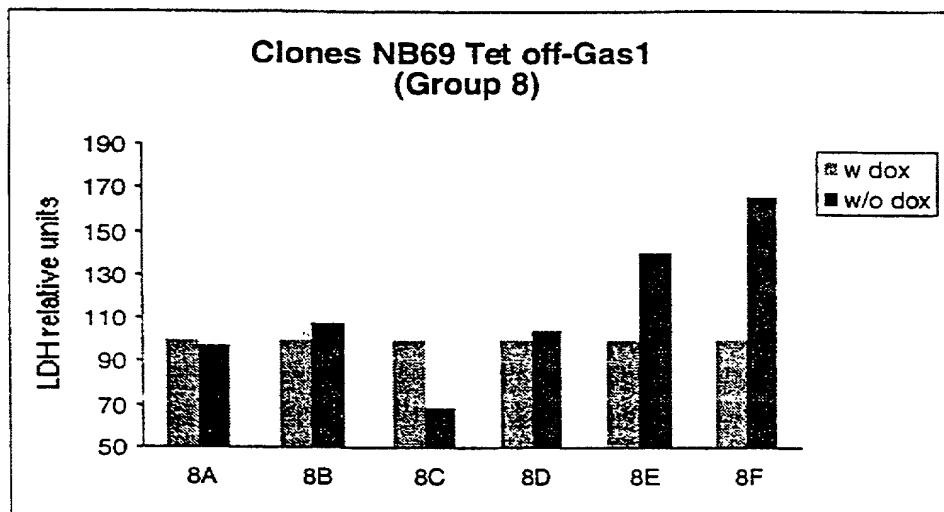


FIG. 2B

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FIGURE 3

A



B

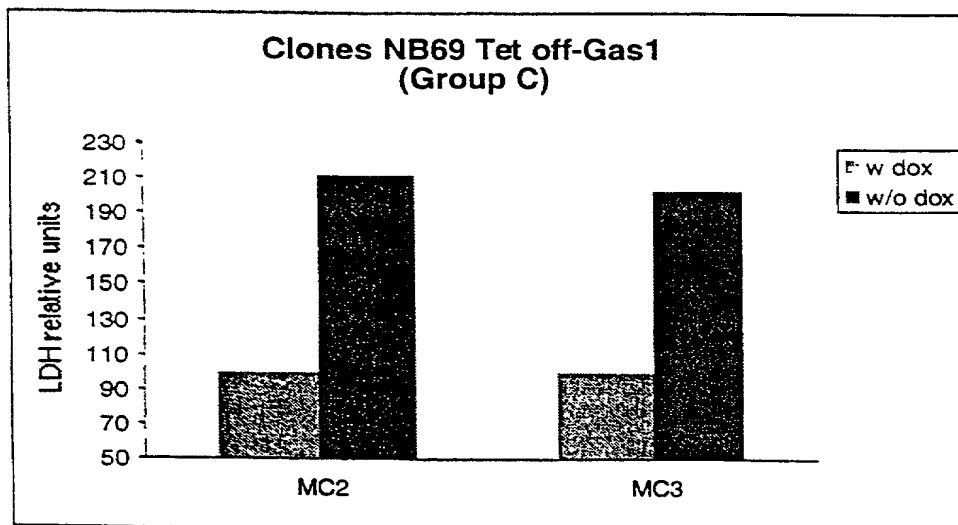
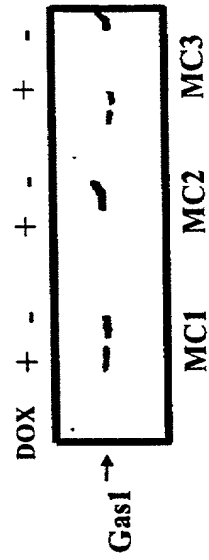


Figure 4



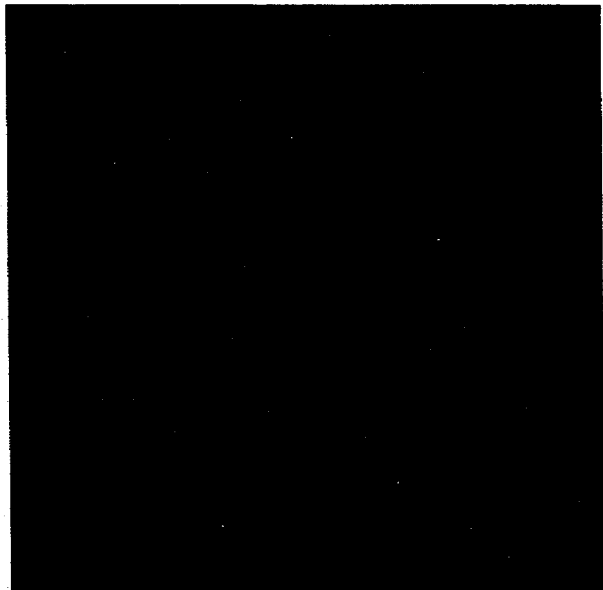
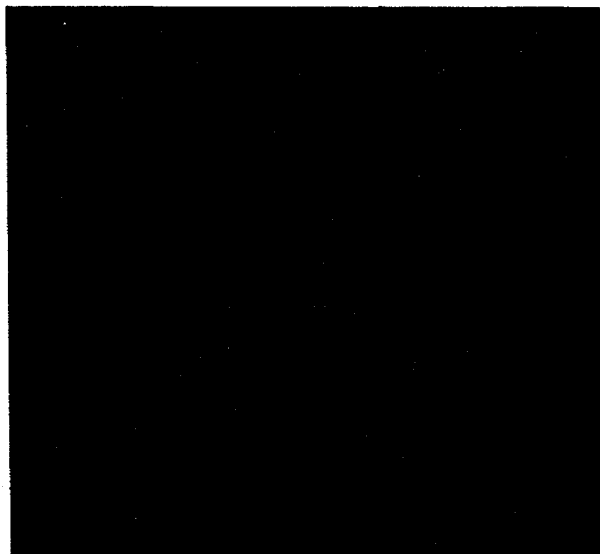


Figure 5

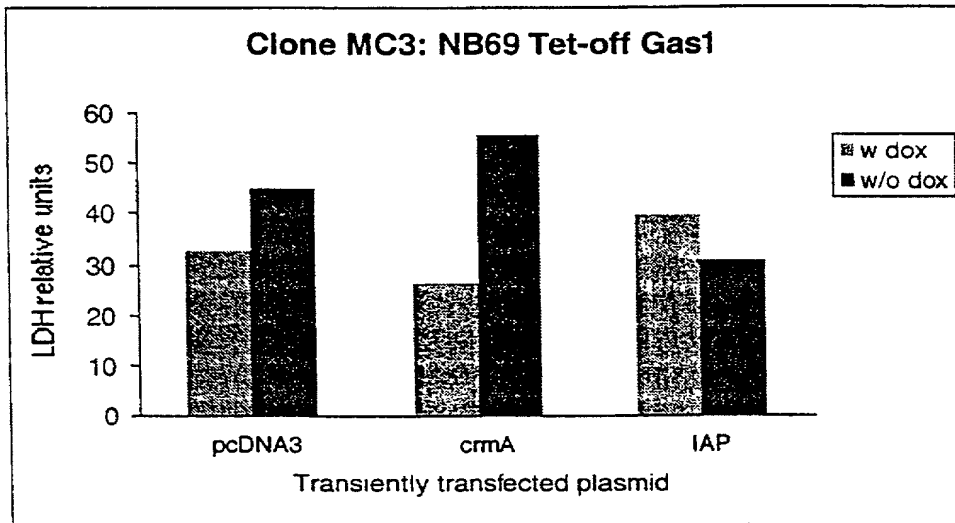


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Figure 6

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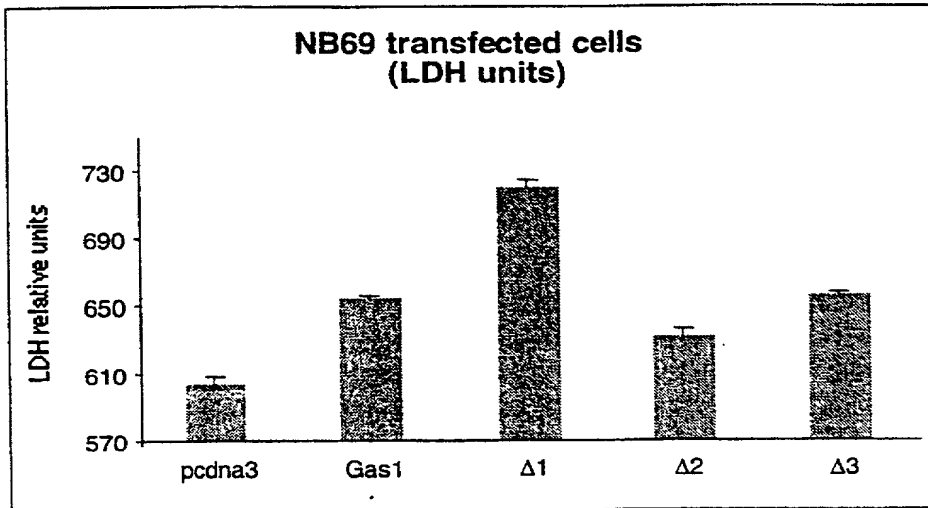
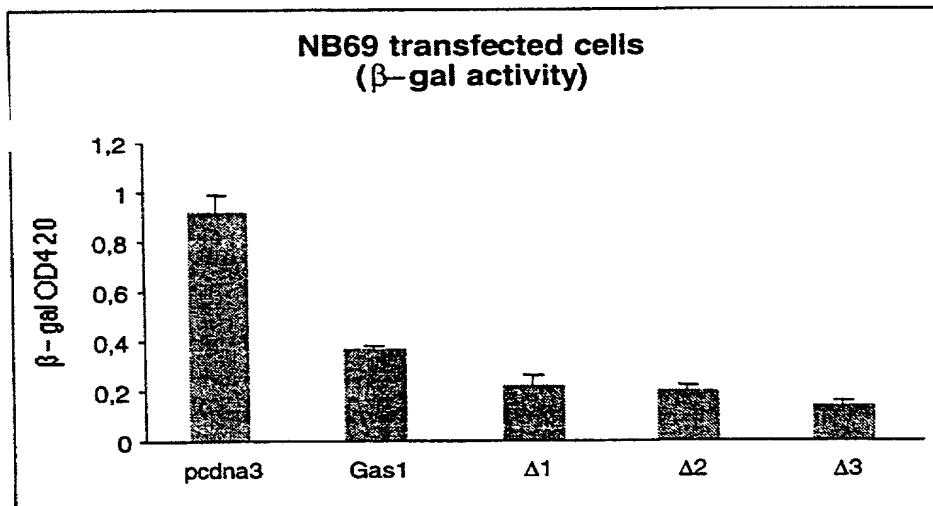
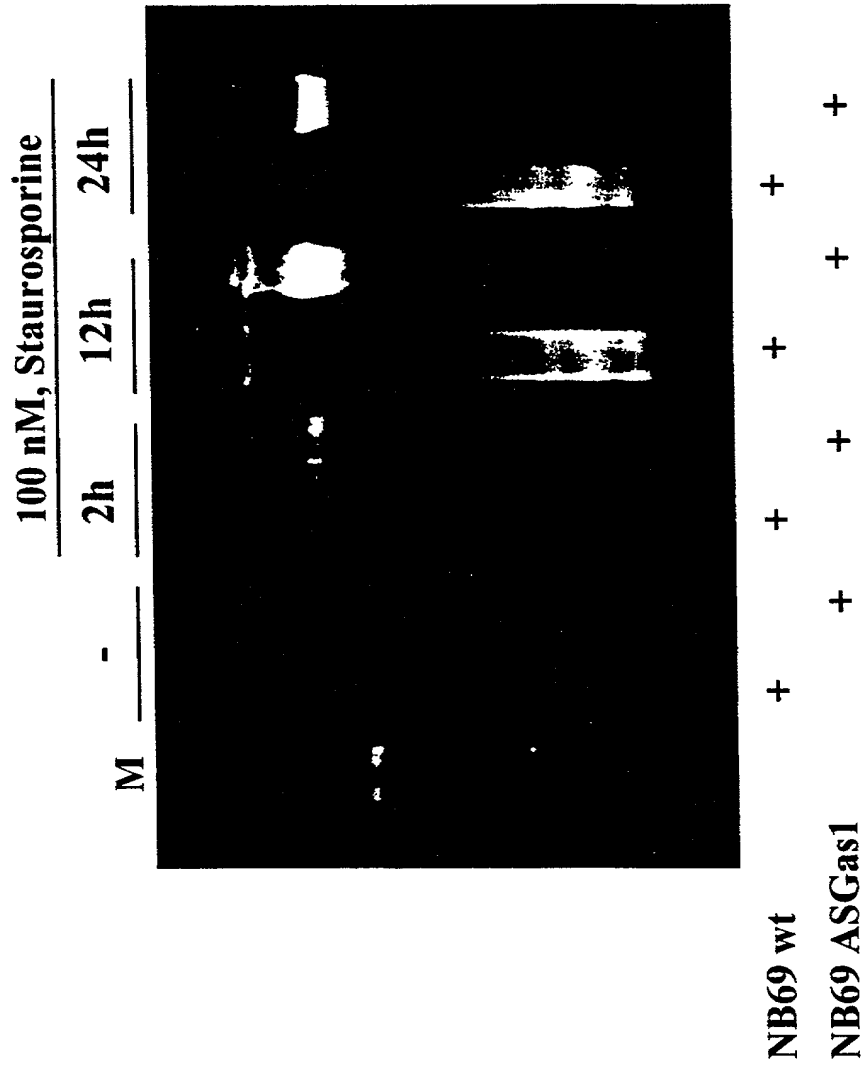
Figure 7**A****B**

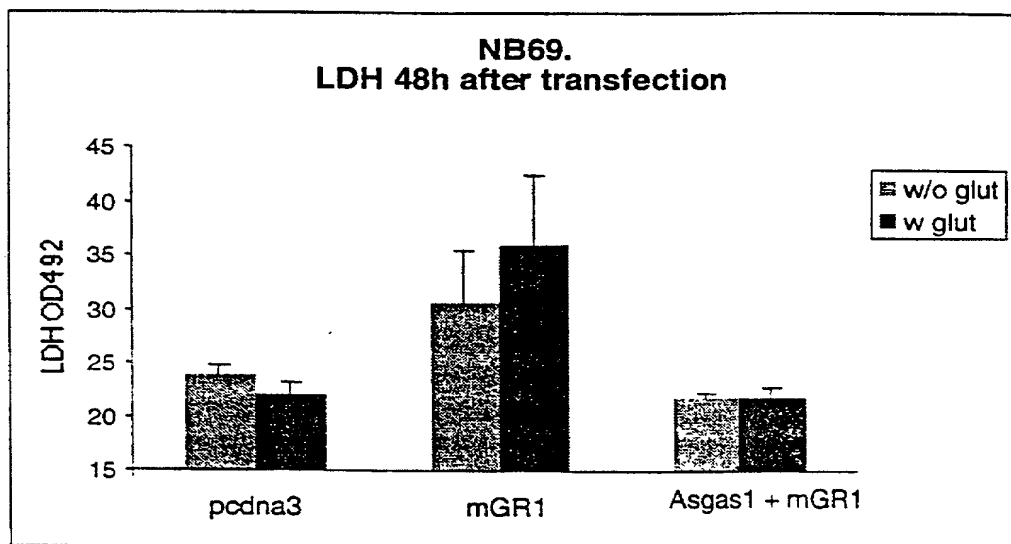
Figure 8



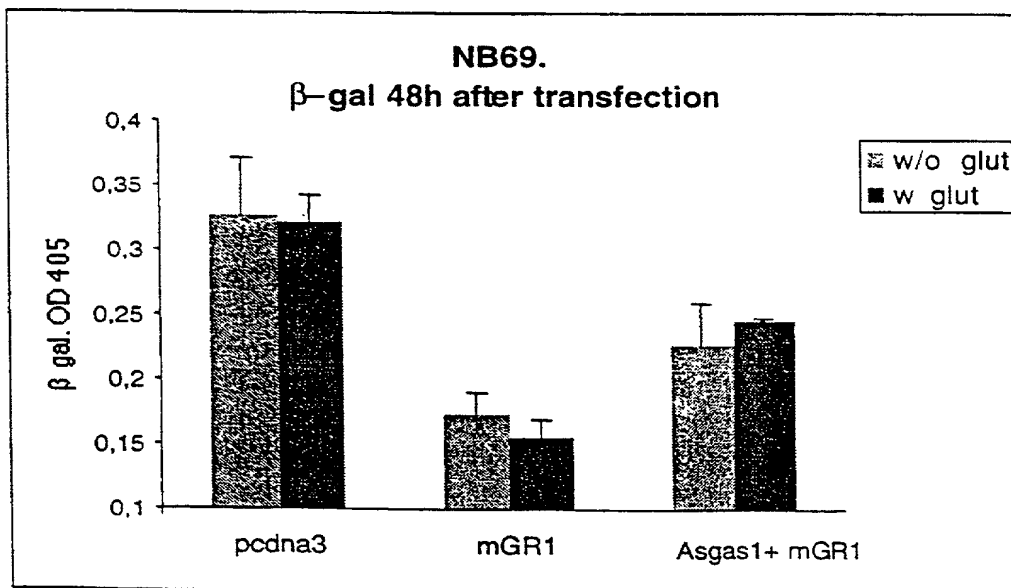
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Figure 9

A



B



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DECLARATION

ADDITIONAL INVENTOR(S) Supplemental Sheet Page 1 of 1

Name of Additional Joint Inventor, if any:		<input type="checkbox"/> A petition has been filed for this unsigned inventor					
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Inventor's Signature						Date	
Residence: City	Madrid	State		Country	Spain	Citizenship	ES
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Post Office Address							
City	Cercedilla / Madrid	State		ZIP	28470	Country	Spain
Name of Additional Joint Inventor, if any:		<input type="checkbox"/> A petition has been filed for this unsigned inventor					
Given Name (first and middle [if any])				Family Name or Surname			
Britt				Mellström			
Inventor's Signature						Date	
Residence: City	Beerse	State		Country	Belgium	Citizenship	SE
Post Office Address		c/o Molecular & Cellular Biology Dept., CNB C.S.I.C., Cantoblanco, E-28049 Madrid, Spain					
Post Office Address							
City	Cercedilla / Madrid	State		ZIP	28470	Country	Spain
Name of Additional Joint Inventor, if any:		<input type="checkbox"/> A petition has been filed for this unsigned inventor					
Given Name (first and middle [if any])				Family Name or Surname			
Inventor's Signature						Date	
Residence: City		State		Country		Citizenship	
Post Office Address							
Post Office Address							
City		State		ZIP		Country	

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DECLARATION FOR UTILITY OR DESIGN PATENT APPLICATION (37 CFR 1.63) <input checked="" type="checkbox"/> Declaration Submitted with Initial Filing OR <input type="checkbox"/> Declaration Submitted after Initial Filing (surcharge (37 CFR 1.16 (e)) required)	Attorney Docket Number	JAB 1526-PCT-USA
	First Named Inventor	Luyten, Walter H. M. L.
	COMPLETE IF KNOWN	
	Application Number	/
	Filing Date	
	Group Art Unit	
	Examiner Name	

As a below named inventor, I hereby declare that:

My residence, post office address, and citizenship are as stated below next to my name

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

GAS1 POLYPEPTIDES

the specification of which

☐ is attached hereto
OR

☒ was filed on (MM/DD/YYYY) 08/21/2000 as United States Application Number or PCT International

Application Number PCT/EP00/08182 and was amended on (MM/DD/YYYY) (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment specifically referred to above

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or of any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Number(s)	Country	Foreign Filing Date (MM/DD/YYYY)	Priority Not Claimed	Certified Copy Attached?	
				YES	NO
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☐ Additional foreign application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto:

I hereby claim the benefit under 35 U.S.C. 119(e) of any United States provisional application(s) listed below.

Application Number(s)	Filing Date (MM/DD/YYYY)	<input type="checkbox"/> Additional provisional application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto

[Page 1 of 2]

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I hereby claim the benefit under 35 U.S.C. 120 of any United States application(s), or 365(c) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

U.S. Parent Application or PCT Parent Number	Parent Filing Date (MM/DD/YYYY)	Parent Patent Number (if applicable)

☐ Additional U.S. or PCT international application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto.

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OR

☒ Registered practitioner(s) name/registration number listed below

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Name	Registration Number	Name	Registration Number
Michael Stark	32,495	Myra McCormack	36,602
Steven P. Berman	24,772	Ellen C. Coletti	34,140
Andrea L. Colby	30,194	Mary A. Appollina	34,087

☐ Additional registered practitioner(s) named on supplemental Registered Practitioner Information sheet PTO/SB/02C attached hereto.

Direct all correspondence to: ☐ Customer Number OR ☒ Correspondence address below

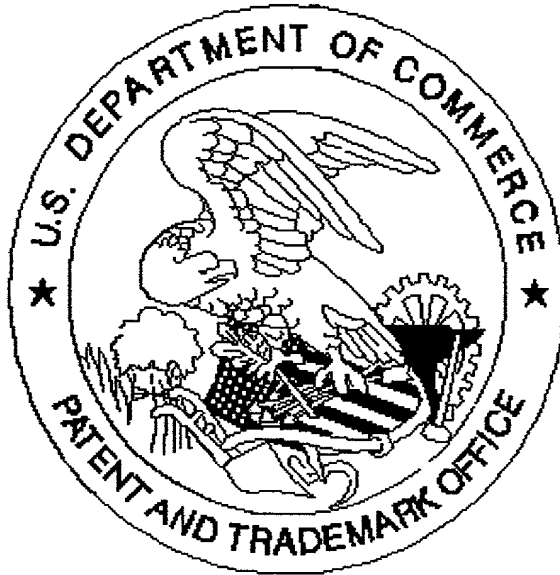
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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Name of Sole or First Inventor:		<input type="checkbox"/> A petition has been filed for this unsigned inventor	
Given Name (first and middle if any)		Family Name or Surname	
Walter Herman Maria Louis		Luyten	
Inventor's Signature	Date		12/12/01
Residence: City	Beerse	State	Country
			Belgium
Citizenship	BE		
Post Office Address	c/o Janssen Pharmaceutica N.V., Turnhoutseweg 30, B-2340 Beerse, Belgium		
Post Office Address			
City	Turnhout	State	ZIP
			2300
Country	Belgium		

☒ Additional inventors are being named on the 1 supplemental Additional Inventor(s) sheet(s) PTO/SB/02A attached hereto

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